Inhibition of melanoma tumor growth *in vivo* by survivin targeting

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A role of apoptosis (programmed cell death) in tumor formation and growth was investigated by targeting the apoptosis inhibitor survivin *in vivo*. Expression of a phosphorylation-defective survivin mutant (Thr^{34}→Ala) triggered apoptosis in several human melanoma cell lines and enhanced cell death induced by the chemotherapeutic drug cisplatin *in vitro*. Conditional expression of survivin Thr^{34}→Ala in YUSAC2 melanoma cells prevented tumor formation upon s.c. injection into CB.17 severe combined immunodeficient-beige mice. When induced in established melanoma tumors, survivin Thr^{34}→Ala inhibited tumor growth by 60–70% and caused increased apoptosis and reduced proliferation of melanoma cells *in vivo*. Manipulation of the antiapoptotic pathway maintained by survivin may be beneficial for cancer therapy.

Regulation of apoptosis (programmed cell death) is critical for normal embryonic development and for homeostasis in adult tissues (1). Dysregulation of this process with increased resistance to cell death is a common feature of malignant cells (2) and represents a significant obstacle to therapy of human cancer (3). Apoptosis resistance in melanoma (4) accounts for its poor response to chemotherapy (5) and has been correlated with enhanced metastasis in animal models (6). The inhibitor of apoptosis (IAP) protein (7) survivin is absent from most adult tissues but notable for its expression in melanoma (8) as well as most other human cancers (9, 10). Survivin expression in tumors has been associated with increased aggressiveness and decreased patient survival (11–15).

Maximally expressed in the G_2/M phase of the cell cycle, survivin physically associates with mitotic spindle microtubules and regulates progression through mitosis (16). Transformed cells are exquisitely sensitive to manipulation of this checkpoint, because interference with survivin expression/function using dominant-negative mutants affecting the baculovirus IAP repeat (BIR; ref. 17) or survivin antisense resulted in dysregulation of mitotic progression (18) and spontaneous apoptosis (8, 19, 20). This response is unique to survivin and not observed with other apoptosis inhibitors potentially contributing to neoplasia, because antisense inhibition of Bcl-2 increased sensitivity to apoptosis but was insufficient in itself to induce cell death (21). Here, we targeted survivin in melanoma *in vivo* by using regulated expression of a phosphorylation-defective Thr^{34}→Ala BIR mutant.

Methods

Survivin Dominant-Negative Mutant and Transient Transfections. The Thr^{34}→Ala mutation was introduced by site-directed mutagenesis into the 1.6-kb human survivin cDNA (9) by using the oligonucleotide 5′-GGCTGCGCCTGCgCCCCGGAGCGGATG-3′ and the GENEEDITOR system (Promega) according to the manufacturer's instructions and cloned into the green fluorescent protein (GFP) marker plasmid pEGFPc1 (CLONTECH).

Human melanoma lines YUSAC2, YUGEN8, and LOX were kindly provided by Ruth Halaban (Yale Univ. School of Medicine, New Haven, CT) and maintained as described (8). Transient transfections were performed and apoptotic index was assessed after 48 h by nuclear morphology by using 4,6-diamidino-2-phenylindole staining as described (8).

Table 1. Induction of apoptosis in melanoma cell lines by expression of survivin Thr^{34} → Ala

Plasmid	YUSAC2	LOX	YUGEN8
GFP-vector	2	11	7
GFP-survivin	12	20	19
GFP-survivin (Thr^{34} → Ala)	40	55	42

The melanoma cell lines YUSAC2, LOX, and YUGEN8 were transfected separately with the indicated GFP-containing plasmids as described previously (8). At 48 h after transfection, cell nuclei were scored morphologically as normal or apoptotic by 4,6-diamidino-2-phenylindole staining and fluorescence microscopy as described previously (8). Data are expressed as percent apoptosis based on counting approximately 100 cells per condition and are the mean of two independent transfection experiments.

Generation of Inducible Transfectants in Melanoma Cells. The wild-type survivin cDNA and the survivin Thr^{34}→Ala mutant were cloned into the *Eco*RI and *Hin*dIII-*Spe*I sites, respectively, of pTet-splice (Stratagene) downstream of the regulatory sequences of the tetracycline (tet)-resistance operon. The plasmid pTA-Neo, containing the tet-controlled transactivator sequence downstream of the tet operon and a neomycin resistance gene, was kindly provided by D. Schatz (Yale Univ. School of Medicine). In this tandem plasmid system, tet prevents transactivator binding to the tet operon and transcription of the transgene; in the absence of tet, the transactivator up-regulates its own transcription and the transgene is expressed (22).

YUSAC2 cells (8) were transfected in six-well plates by the addition of 0.8 μg of pTet-splice containing either the wild-type survivin cDNA or the survivin Thr^{34}→Ala mutant, 0.8 μg of pTA-Neo, 0.5 μg of tet (Sigma), and 5 μl of Lipofectamine (Life Technologies, Gaithersburg, MD) per well. After 9 h, the transfection medium was aspirated and replaced with serum-containing medium in the presence of 0.5 μg/ml tet. Forty-eight h after transfection, cells were trypsinized, washed, and replated at low density in 15 × 150-mm plates in fresh medium containing 1.5 mg/ml G418 (Life Technologies), 2 mM sodium hydroxide, and 0.5 μg/ml tet. This selection medium was changed every 6 days, and after 3 weeks colonies were transferred to U-bottom microtiter wells for expansion and screening on the basis of tet-regulated differential growth (survivin Thr^{34}→Ala) or induction of survivin-immunoreactive material (wild-type trans-

Abbreviations: IAP, inhibitor of apoptosis; tet, tetracycline; BIR, baculovirus IAP repeat; GFP, green fluorescent protein; TUNEL, terminal deoxynucleotidyltransferase-mediated UTP end labeling.

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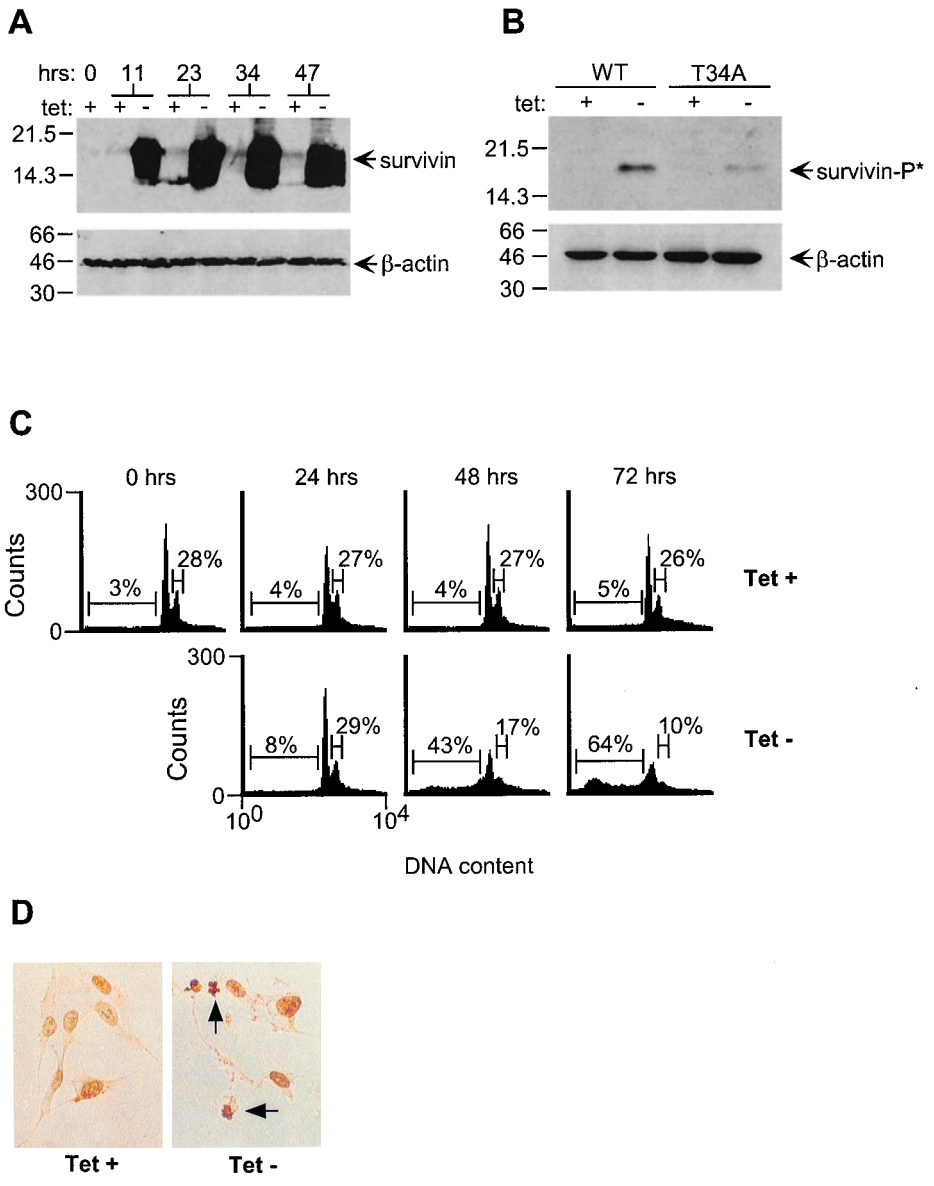

Fig. 1. Characterization of tet-regulated induction of survivin Thr^{34}→Ala in YUSAC2/T34A-C4 cells. (*A*) YUSAC2/T34A-C4 cells were cultured in the presence or absence of tet (0.5 μg/ml) and harvested at the indicated time intervals, and lysates (100 μg/lane) were subjected to Western blotting with antibodies to survivin (*Upper*) or β-actin (*Lower*). (*B*) YUSAC2/WT or YUSAC2/T34A-C4 cells were cultured for 24 h in the presence or absence of tet, and lysates (50 μg/lane) were analyzed by Western blotting with an antibody to phosphorylated T34* (α-survivinT34*) (*Upper*) or β-actin (*Lower*). For *A* and *B*, molecular mass markers in kDa are indicated at the left of each image. (*C*) YUSAC2/T34A-C4 cells were cultured in the presence or absence of tet for the indicated time intervals, and nonadherent and adherent cells were pooled and analyzed for DNA content by propidium iodide staining and flow cytometry. Percentages shown are for the sub-G_1 fraction (left marker), corresponding to apoptotic cells, and the G_2/M fraction (right marker), corresponding to mitotic cells. (*D*) YUSAC2/T34A-C4 cells were cultured for 48 h in the presence or absence of tet. Arrows indicate TUNEL-reactive cells with apoptotic morphology.

fectants). Clones stably transfected with the wild-type survivin (YUSAC2/WT) and survivin Thr^{34}→Ala (YUSAC2/T34A-C4 and YUSAC2/T34A-E5) were isolated and maintained in selection medium in the presence of G418 and tet.

Expression and Function of Survivin Thr^{34}→Ala *in Vitro*. Western blotting was performed as described (8) by using a polyclonal antibody reactive against both native and mutant (Thr^{34}→Ala) survivin. A novel rabbit polyclonal antibody was raised against the survivin peptide L^{28}EGCACT*PERMAEAGFI^{44} containing phosphorylated Thr^{34} (T*). The serum was precleared over a nonphosphorylated peptide-Sepharose column, and unbound material was affinity-purified over a phosphorylated peptide-Sepharose column. This antibody to phosphorylated survivin Thr^{34} (α-survivinT34*) recognized wild-type survivin after *in vitro* phosphorylation by baculovirus-expressed p34^{cdc2}-cyclin B1, but not unphosphorylated wild-type survivin or survivin Thr^{34}→Ala after incubation with p34^{cdc2}-cyclin B1 (25). Blots were stained with α-survivinT34* (10 μg/ml) overnight at 4°C. For DNA content analysis, both nonadherent and adherent cells were recovered and pooled. Cells then were fixed, permeabilized, and stained with propidium iodide as described (8). Percentages for the sub-G_1 fraction corresponding to apoptotic cells, and the G_2/M fraction, corresponding to mitotic cells, were derived from histograms obtained with CELL QUEST software (Becton Dickinson). For terminal deoxynucleotidyltransferase-mediated UTP end labeling (TUNEL) analysis of cultured cells, cells were plated on coverslips treated with 2% gelatin (Sigma)

and incubated 48 h in the presence or absence of tet. TUNEL staining was carried out by using the ApopTag kit (Intergen, Purchase, NY) according to the manufacturer's instructions as described (8). Cisplatin (Sigma) was solubilized (30 mM) in dimethylformamide and stored at 4°C.

Tumor Formation in CB.17 Mice. We determined that YUSAC2 cells consistently form localized tumors in 6- to 8-week-old CB.17 SCID/beige mice (Taconic Farms, Germantown, NY) approximately 2–3 weeks after s.c. injection of 2–3 $\times$ 10^6 cells. We have monitored animals for up to 4 months, and neither mortality nor gross metastasis is associated with increasing tumor size (up to 5,000 mm^3) or ulceration. One day before injection, mice were shaved on the right flank, and the regular drinking water was replaced with 5% sucrose alone or containing 100 μg/ml tet as described (22). Cells were harvested in log-phase growth, washed twice in PBS, resuspended in PBS (12 $\times$ 10^6 cells/ml), and injected (0.25 ml, 3 $\times$ 10^6 cells) s.c. The drinking water was changed every 2–3 days. Tumor size was determined by the product of two perpendicular diameters and the height above the skin surface.

Analysis of Apoptosis and Proliferation *in Vivo*. Apoptotic cells *in vivo* were identified by TUNEL staining as described above. An apoptotic index was obtained by counting the average number of TUNEL-positive cells in 12 low-power ($\times$100) fields, each containing approximately 4,000 cells. For determination of proliferating cells *in vivo*, mice were injected i.p. with 50 mg/kg BrdUrd (Sigma) in PBS, and tumors were excised 2 h later. Tissue sections were stained for BrdUrd by using a kit (Zymed) according to the manufacturer's instructions. A proliferative index was obtained by counting the average number of BrdUrd-positive cells in 12 high-power ($\times$400) fields, each containing approximately 1,000 cells.

Reestablishment of Tumor Cell Lines *in Vitro*. Tumors were excised surgically and skin and s.c. tissues were dissected away. Tumors were cut into small pieces with a sterile razor blade and dissociated into a single-cell suspension by vortexing in PBS. After removal of insoluble debris, cells were washed twice in PBS, resuspended in selection medium, and cultured for two to three passages.

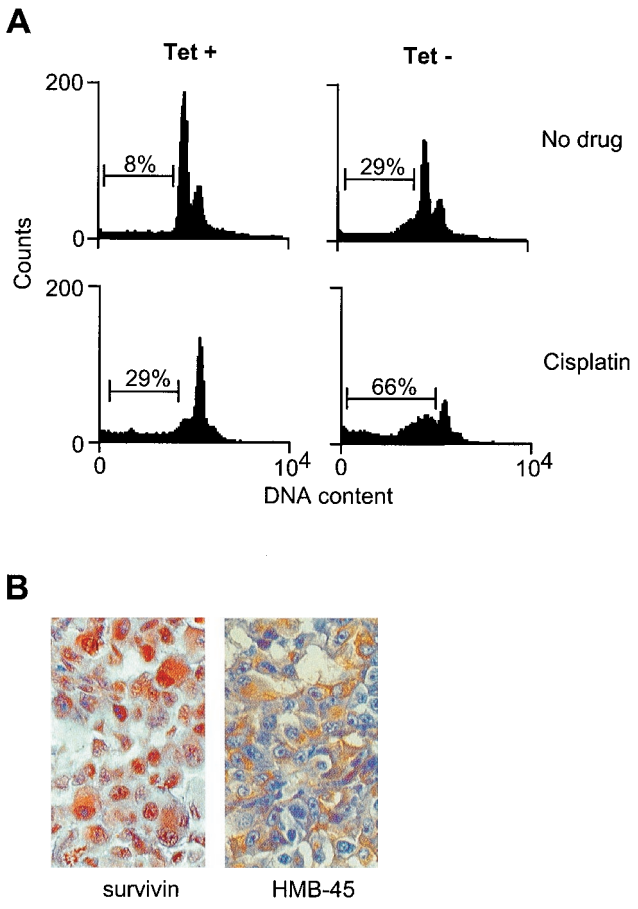

Fig. 2. Enhancement of cisplatin-induced apoptosis by expression of survivin Thr^{34}→Ala, and histology of YUSAC2 tumors *in vivo*. (*A*) YUSAC2/T34A-C4 cells were cultured for 3 days in the presence or absence of tet and 3 μM cisplatin, as indicated, and stained for DNA content as described in Fig. 1. Percentages shown are for the sub-G_1 fraction (left marker), corresponding to apoptotic cells. (*B*) Sections of (untransfected) YUSAC2 tumor ($\times$400) were stained with antibodies to survivin or HMB-45 (Zymed) as indicated.

Results and Discussion

A survivin Thr^{34}→Ala mutation, which abolishes a phosphorylation site for the main mitotic kinase p34^{cdc2}-cyclin B1, caused spontaneous apoptosis (i.e., a dominant-negative effect) when overexpressed in HeLa carcinoma cells (25). Transfection of three different human melanoma cell lines (8) with GFP conjugates of survivin Thr^{34}→Ala also resulted in morphologic signs of apoptosis including chromatin condensation and DNA fragmentation (Table 1). By contrast, GFP (wild-type)-survivin did not affect melanoma cell viability (Table 1).

One of these melanoma lines, YUSAC2, was stably transfected with survivin Thr^{34}→Ala or the wild-type survivin cDNA under the control of a tet-regulated ("tet-off") promoter system (22). Upon withdrawal of tet from the culture medium, subclone YUSAC2/T34A-C4 strongly expressed a 16.5-kDa induced survivin band by Western blotting (Fig. 1*A*). By contrast, no modulation of survivin expression was observed when tet was present in the culture medium (Fig. 1*A*). Similar results of tet-regulated induction of survivin Thr^{34}→Ala were obtained with another subclone, YUSAC2/T34A-E5 (not shown). Reactivity by Western blotting with phosphosurvivin-specific antibody (α-survivinT34*) was considerably reduced in tet− cultures of YUSAC2/T34A-C4 as compared with tet− cultures of YUSAC2/WT cells (Fig. 1*B*). Tet-regulated expression of the Thr^{34}→Ala mutant resulted in a progressive, time-dependent accumulation of apoptotic cells with hypodiploid (sub-G_1) DNA content and coincident loss of mitotic (G_2/M) cells, as assessed by propidium iodide staining and flow cytometry (Fig. 1*C*). Tet-deprived YUSAC2/T34A-C4 cells exhibited apoptotic nuclear morphology and stained intensely for internucleosomal DNA fragmentation by TUNEL (Fig. 1*D*). By contrast, YUSAC2/T34A-C4 cells cultured in the presence of tet demonstrated normal mitotic progression (Fig. 1*C*) and did not stain for TUNEL (Fig. 1*D*). In addition to spontaneous apoptosis, induction of survivin Thr^{34}→Ala increased the sensitivity of YUSAC2 cells to the chemotherapeutic drug cisplatin. A 3-day culture of YUSAC2/T34A-C4 cells in the presence of tet and cisplatin resulted in a population of both apoptotic cells and cells arrested in G_2/M (Fig. 2*A*), consistent with previous reports (23) of cisplatin-treated melanoma cells. Cultures treated with cisplatin and deprived of tet revealed a 2.2-fold increase in apoptotic cells, as compared with either treatment alone (Fig. 2*A*).

Next, we asked whether interference with survivin function by regulated expression of survivin Thr^{34}→Ala mutant could block melanoma tumor formation in CB.17 severe combined immunodeficient-beige mice. Untransfected YUSAC2 cells readily formed localized nodular amelanotic tumors in mice composed

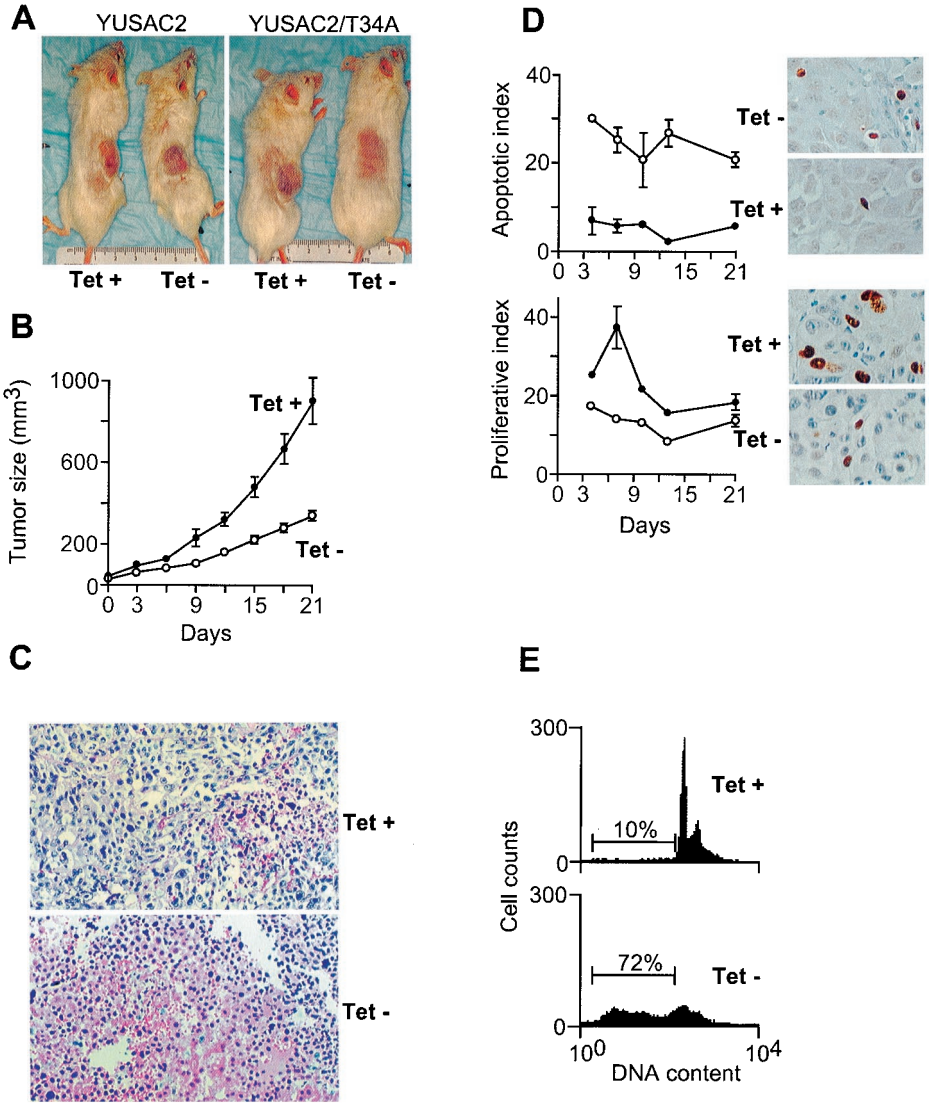

Fig. 3. Effect of survivin Thr[34]→Ala mutant in melanoma tumors *in vivo* and tet-regulated apoptosis of tumor lines reestablished *in vitro*. (*A*) Untransfected YUSAC2 cells (*Left*) or YUSAC2/T34A-C4 cells (*Right*) were injected s.c. into CB.17 mice, and tet was added (left side of images) or withheld (right side of images) from the drinking water as indicated. Photographs were taken of representative mice 8 weeks after injection. (*B*) YUSAC2/T34A-C4 tumors were established in 30 animals maintained on tet. When tumors became apparent (day 0), tet was maintained in the drinking water of 10 animals (tet+, ●) and withheld from 20 animals (tet−, ○), and tumors were monitored for 3 weeks. Bars = SD, $P < 0.0001$ for days 12–21 by two-tailed *t* test. (*C*) Histology of established YUSAC2/T34A-C4 tumors from tet+ (*Upper*) and tet− (*Lower*) animals after 8 weeks by hematoxylin/eosin staining (×100). (*D*) YUSAC2/T34A-C4 tumors were established in animals on tet, and when tumors became apparent (day 0), tet was maintained in the drinking water of half the animals (tet+, ●) and withheld from the others (tet−, ○). Tumors were excised on days 4, 7, 10, 13, and 21 and subjected to TUNEL and BrdUrd staining for respective determination of apoptotic (upper graph) and proliferative (lower graph) indices. Bars = SEM from 2 animals for days 4 and 13, 4 animals for days 7 and 10, and 10 animals for day 21. Adjacent images are representative fields (×400) from day 7 tumors ± tet as indicated. (*E*) Cell lines from several YUSAC2/T34A-C4 tumors slowly growing *in vivo* after withdrawal of tet were reestablished *in vitro*. The DNA content analysis for a representative line after culturing for 72 h in the presence (*Upper*) or absence (*Lower*) of tet is shown. The marker and percentages indicate the sub-G_1 fraction, corresponding to apoptotic cells.

of sheets of large, epithelioid malignant cells that stained positively for survivin and HMB-45, a marker of human melanoma cells (24; Fig. 2*B*). Tumor formation by YUSAC2 cells was not affected by the presence or absence of tet in the drinking water (Fig. 3*A*; Table 2). By contrast, YUSAC2/T34A-C4 cells did not form tumors in 13 of 15 (87%) animals when tet was withheld from the drinking water (Fig. 3*A*; Table 2). All of these animals remained tumor-free for an additional 3-month observation period. The two tumors that formed in tet-deprived animals were considerably smaller in size and appeared with a markedly delayed onset compared with those in animals given tet (Table 2). The slightly smaller size of tumors formed in the presence of tet by survivin Thr[34]→Ala subclones compared with untransfected YUSAC2 cells (Table 2) may be due to minimal leakiness of the promoter system *in vivo*. Prevention of melanoma tumor formation *in vivo* by survivin Thr[34]→Ala also was obtained with subclone YUSAC2/T34A-CE5 (Table 2).

We next investigated whether expression of survivin Thr[34]→Ala could affect the growth of already established melanoma tumors. For these experiments, 30 mice were injected with YUSAC2/T34A-C4 cells and tet was provided to permit tumor formation. After 3 weeks, tumors became palpable (20–50 mm^3) and tet was withheld from the drinking water of 20 animals. Tumor sizes in all of the animals then were monitored for an additional 3 weeks. Tumors of animals maintained on tet exhibited exponential growth during the 3-week observation

Table 2. Prevention of tumor formation by tet-regulated expression of survivin Thr^{34} → Ala

Cell line*	Transgene†	Tet‡	Tumors at 4 weeks§		Tumors at 8 weeks§	
YUSAC2	None	+	5/5	295 ± 129	5/5	2,580 ± 1,426
YUSAC2	None	−	5/5	358 ± 283	5/5	2,086 ± 1,228
YUSAC2/T34A-C4	Survivin Thr^{34} → Ala	+	3/4	181 ± 18	4/4	1,533 ± 1,019
YUSAC2/T34A-C4	Survivin Thr^{34} → Ala	−	0/15	—	2/15	278 ± 116
YUSAC2/T34A-E5	Survivin Thr^{34} → Ala	+	3/4	132 ± 115	3/4	924 ± 710
YUSAC2/T34A-E5	Survivin Thr^{34} → Ala	−	0/5	—	0/5	—

*CB.17 mice were injected s.c. with 3×10^6 cells of the indicated cell line, and tumors were measured 4 and 8 weeks later.
†In the "tet-off" promoter system used, the transgene is expressed only in the absence of tet.
‡Tet was added (+, 100 μg/ml) or withheld (−) from the drinking water as indicated.
§Incidence and mean tumor size (mm^3) ± SD of tumors formed at the indicated observation point.

period (Fig. 3*B*). By contrast, withdrawal of tet in established tumors was associated with a significant ($P < 0.0001$) reduction (60–70%) in growth rate (Fig. 3*B*).

The long-term effects of survivin targeting in established melanoma tumors were investigated in 10 additional animals in which tet had been withdrawn. At 8 weeks, animals were killed and histologic examination of these tumors was performed. Tumors in animals deprived of tet contained massive areas of necrosis, compared with minimal loss of cell viability in tumors of animals maintained on tet (Fig. 3*C*).

Next, we examined whether the decreased growth rate in survivin-targeted tumors was associated with an increased rate of spontaneous apoptosis in melanoma cells. Whereas tumors from animals maintained on tet contained low numbers of apoptotic cells, tumors from animals deprived of tet revealed consistently increased numbers of TUNEL-positive cells throughout the 21-day observation period of predicted expression of survivin Thr^{34}→Ala (Fig. 3*D*). In control experiments, no cells were stained for TUNEL in the absence of terminal deoxynucleotidyl transferase (not shown).

Given the role of survivin in controlling apoptosis (16) and ploidy at cell division (18), we next examined the rate of melanoma cell proliferation by BrdUrd incorporation in the absence or presence of survivin targeting. Whereas tumors from animals maintained on tet demonstrated an initial burst of proliferative activity, tumors from animals deprived of tet revealed no proliferative burst and consistently lower levels of BrdUrd-labeled cells throughout the observation period (Fig. 3*D*). No cells were stained for BrdUrd in the absence of BrdUrd injection (not shown).

Finally, we wished to confirm that the suppressive effect of survivin targeting on tumor growth and viability *in vivo* could be attributed to spontaneous apoptosis induced by tet-regulated expression of survivin Thr^{34}→Ala. For these experiments, cell lines were reestablished *in vitro* from several melanoma tumors excised from animals deprived of tet and analyzed for tet-regulated induction of apoptosis *in vitro*. In all cases, these cells retained tet responsiveness as removal of tet from the culture medium was associated with generation of hypodiploid (apoptotic) cells by DNA content analysis (Fig. 3*E*) and in agreement with the data presented above (Fig. 1). The persistence of some viable cells in tet-deprived tumors may reflect an inability to remove tet completely from the animal and achieve maximal transgene expression *in vivo*. In addition, inhibition of survivin may not eliminate nondividing cells given its selective action during the G_2/M phase of the cell cycle (16).

In summary, we have used a tet-regulated molecular antagonist of survivin, i.e., a dominant-negative mutant, to interfere with the apoptotic balance in melanoma tumors *in vivo*. The Thr^{34} residue in survivin corresponds to a unique p34^{cdc2} phosphorylation site, and mutagenesis of Thr^{34}→Ala completely suppressed survivin phosphorylation by p34^{cdc2}-cyclin B1 *in vitro* and *in vivo* (25). Based on the survivin crystal structure, Thr^{34} appears ideally positioned to modulate protein recognition potentially mediated by the survivin BIR in a phosphorylation-dependent manner (17). The ability of survivin Thr^{34}→Ala mutant to localize to mitotic spindle microtubules and associate with p34^{cdc2}-cyclin B1 (25) suggests that its dominant-negative affect may reflect interference with phosphorylation of endogenous survivin. Consistent with this prediction, tet-regulated expression of survivin Thr^{34}→Ala resulted in considerable inhibition of survivin phosphorylation on Thr^{34}, as determined by Western blotting with a T34* phosphorylation-specific antibody. Functionally, this resulted in spontaneous apoptosis of melanoma cells *in vitro* and *in vivo*, enhanced sensitivity to a chemotherapeutic drug (cisplatin), suppression of *de novo* melanoma tumor formation, and growth inhibition of already established melanoma tumors. Consistent with a potential effect on the apoptosis balance at mitosis, expression of survivin Thr^{34}→Ala also resulted in loss of cells with G_2/M DNA content *in vitro* and decreased proliferation of melanoma cells *in vivo*. Combined with the selective overexpression of survivin in cancer but not in normal tissues (9, 10), these data suggest that manipulation of the survivin pathway alone or with chemotherapy may be therapeutically useful in the treatment of melanoma and other malignancies.

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1. Thornberry, N. A. & Lazebnik, Y. (1998) *Science* **281,** 1312–1316.
2. Reed, J. C. (1999) *J. Clin. Oncol.* **17,** 2941–2953.
3. Rudin, C. M. & Thompson, C. B. (1997) *Annu. Rev. Med.* **48,** 267–281.
4. Li, G., Tang, L., Zhou, X., Tron, V. & Ho, V. (1998) *Melanoma Res.* **8,** 17–28.
5. Koh, H. K. (1991) *N. Engl. J. Med.* **325,** 171–182.
6. Glinsky, G. V., Glinsky, V. V., Ivanova, A. B. & Hueser, C. J. (1997) *Cancer Lett.* **115,** 185–193.
7. Deveraux, Q. L. & Reed, J. C. (1999) *Genes Dev.* **13,** 239–252.
8. Grossman, D., McNiff, J. M., Li, F. & Altieri, D. C. (1999) *J. Invest. Dermatol.* **113,** 1076–1081.
9. Ambrosini, G., Adida, C. & Altieri, D. C. (1997) *Nat. Med.* **3,** 917–921.
10. Velculescu, V. E., Madden, S. L., Zhang, L., Lash, A. E., Yu, J., Rago, C., Lal, A., Wang, C. J., Beaudry, G. A., Ciriello, K. M., *et al.* (1999) *Nat. Genet.* **23,** 387–388.

11. Adida, C., Berrebi, D., Peuchmaur, M., Reyes-Mugica, M. & Altieri, D. C. (1998) *Lancet* **351,** 882–883.
12. Kawasaki, H., Altieri, D. C., Lu, C.-D., Toyoda, M., Tenjo, T. & Tanigawa, N. (1998) *Cancer Res.* **58,** 5071–5074.
13. Monzo, M., Rosell, R., Felip, E., Astudillo, J., Sanchez, J. J., Maestre, J., Martin, C., Font, A., Barnadas, A. & Abad, A. (1999) *J. Clin. Oncol.* **17,** 2100–2104.
14. Swana, H. S., Grossman, D., Anthony, J. N., Weiss, R. M. & Altieri, D. C. (1999) *N. Engl. J. Med.* **341,** 452–453.
15. Tanaka, K., Iwamoto, S., Gon, G., Nohara, T., Iwamoto, M. & Tanigawa, N. (2000) *Clin. Cancer Res.* **6,** 127–134.
16. Li, F., Ambrosini, G., Chu, E. Y., Plescia, J., Tognin, S., Marchisio, P. C. & Altieri, D. C. (1998) *Nature (London)* **396,** 580–584.
17. Muchmore, S. W., Chen, J., Jakob, C., Zakula, D., Matayoshi, E. D., Wu, W., Zhang, H., Li, F., Ng, S. C. & Altieri, D. C. (2000) *Mol. Cell* **6,** 173–182.
18. Li, F., Ackermann, E. J., Bennett, C. F., Rothermel, A. L., Plescia, J., Tognin, S., Villa, A., Marchisio, P. C. & Altieri, D. C. (1999) *Nat. Cell Biol.* **1,** 461–466.
19. Grossman, D., McNiff, J. M., Li, F. & Altieri, D. C. (1999) *Lab. Invest.* **79,** 1121–1126.
20. Olie, R. A., Simoes-Wust, A. P., Baumann, B., Leech, S. H., Fabbro, D., Stahel, R. A. & Zangemeister-Wittke, U. (2000) *Cancer Res.* **60,** 2805–2809.
21. Jansen, B., Schlagbauer-Wadl, H., Brown, B. D., Bryan, R. N., van Elsas, A., Muller, M., Wolff, K., Eichler, H. G. & Pehamberger, H. (1998) *Nat. Med.* **4,** 232–234.
22. Shockett, P., Difilippantonio, M., Hellman, N. & Schatz, D. G. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 6522–6526.
23. Citro, G., D'Agnano, I., Leonetti, C., Perini, R., Bucci, B., Zon, G., Calabretta, B. & Zupi, G. (1998) *Cancer Res.* **58,** 283–289.
24. Kikuchi, A., Shimizu, H. & Nishikawa, T. (1996) *Br. J. Dermatol.* **135,** 400–405.
25. O'Connor, D. S., Grossman, D., Mescia, J., Li, F., Zhang, H., Villa, A., Tognin, S., Marchisio, P. C. & Altieri, D. C. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 13103–13107. (First Published November 7, 2000; 10.1073/pnas.240390697)