Acute Ablation of Survivin Uncovers p53-dependent Mitotic Checkpoint Functions and Control of Mitochondrial Apoptosis*

Received for publication, August 26, 2003, and in revised form, October 22, 2003
Published, JBC Papers in Press, October 27, 2003, DOI 10.1074/jbc.M309479200

Elena Beltrami‡, Janet Plescia§, John C. Wilkinson§§, Colin S. Duckett§§, and Dario C. Altieri¶¶

From the ¶Department of Cancer Biology and the Cancer Center, University of Massachusetts Medical School, Worcester, Massachusetts 01605 and the §Department of Pathology and Department of Internal Medicine, University of Michigan, Ann Arbor, Michigan 48109

Survivin is a member of the Inhibitor of Apoptosis gene family that has been implicated in cell division and suppression of apoptosis. Here, we show that preferential ablation of the nuclear pool of survivin by RNA interference produces a mitotic arrest followed by re-entry into the cell cycle and polyploidy. Survivin ablation causes multiple centrosomal defects, aberrant multipolar spindle formation, and chromatin missegregation, and these phenotypes are exacerbated by loss of the cell cycle regulator, p21(Waf1/Cip1) in p21−/− cells. The mitotic checkpoint activated by loss of survivin is mediated by induction of p53 and associated with increased expression of its down-stream targets, p21(Waf1/Cip1). Accordingly, p53−/− cells exhibit reduced mitotic arrest and enhanced polyploidy upon survivin ablation as compared with their p53+/− counterparts. Partial reduction of the cytosolic pool of survivin by RNA interference sensitizes cells to ultraviolet B-mediated apoptosis and results in enhanced caspase-9 proteolytic cleavage, whereas complete ablation of cytosolic survivin causes loss of mitochondrial membrane potential and spontaneous apoptosis. These data demonstrate that survivin has separable checkpoint functions at multiple phases of mitosis and in the control of mitotic-dependent apoptosis.

Programmed cell death, or apoptosis, is essential for embryonic development and the homeostasis of adult organisms (1). This involves a dynamic coupling of apoptotic pathways to checkpoint mechanisms that survey cell cycle transitions and eliminate potentially dangerous cells before they progress through mitosis (2). Molecules functioning at the checkpoint interface between cell proliferation and cell survival, such as p53 (3), are essential to preserve genomic integrity, and their deregulation is a hallmark of cancer (4). Survivin is a member of the Inhibitor of Apoptosis (IAP) gene family (5) that becomes dramatically overexpressed in cancer in response to oncogene activation (6), loss of p53 (7, 8) or deregulated transcription of patterning pathways, i.e. Wnt (9), and frequently heralds unfavorable disease outcome (10). Although the function of survivin has been dubbed as controversial in the past (11), available published evidence has shown remarkable agreement in assigning a dual role of this protein in mitotic control and inhibition of apoptosis. As early as 1998, studies of antisense inhibition of survivin provided clear evidence for separate defects in mitotic progression and cell death (12), an observation independently confirmed by several groups using different gene targeting strategies and validated in transgenic animals, in vivo (10).

Despite the wealth of information accumulated on survivin, precisely how this pathway couples to the cell death and cell division machineries has not been completely elucidated. Structural data and analysis of caspase catalytic activity indicate that the cytoprotective function of survivin may not conform to the paradigm of other IAP family proteins as endogenous caspase inhibitors (13). Similarly, a proposed evolutionary conserved role of survivin in late stage mitosis, or cytokinesis (14, 15), has been contrasted by data suggesting a broader role at cell division, involving centrosome function (16), spindle checkpoint (17), and microtubule dynamics (17). Adding further complexity, survivin has been shown to exist in immunochemically distinct subcellular pools that may exert different cellular functions (18), and two alternatively spliced survivin isoforms have been described (19).

In an effort to position more accurately the survivin pathway in mitotic control and cell death, we have now taken an RNA interference (RNAi) approach (20) to acutely knock down survivin expression in genetically defined tumor cell types. We obtained clear evidence that survivin is embedded in a p53-dependent mitotic checkpoint (3) that affects multiple phases of mitosis and directly couples to upstream events in mitochon-}

MATERIALS AND METHODS
dsRNA Design—Two different dsRNA were identified in the survivin mRNA to selectively target exon 1 (GGACCCCGGAGUCUCUACA, sequence S1) or exon 3 (GAGGCGAGACGAAAAUGGC, sequence S4). The S1 and S4 dsRNA oligonucleotides as well as a control dsRNA sequence (VIII) were purchased from Dharmacon. The S1 sequence is identical to the dsRNA oligonucleotide recently reported by Carvalho et al. (21).

Cell Culture and Transfections—Cervical carcinoma HeLa cells were maintained in culture in DMEM supplemented with 10% FCS, 100 units/ml penicillin, and 100 µg/ml streptomycin (all from Invitrogen) in 5% CO2 at 37°C. Colo-rectal carcinoma HCT-116 p21+/− or p21−/− or p53+/− or p53−/− were the kind gift of Dr. Bert Vogelstein (Johns Hopkins University) and were maintained in culture in McCoy’s medium supplemented with 10% FCS, 100 units/ml penicillin, and 100 µg/ml streptomycin in 5% CO2 at 37°C. For Western blotting or flow cytometry experiments, HeLa or HCT-116 cells were seeded in complete medium in 6-well plates at a density of 1 x 10⁵ cells per well. Cells were transfected after 24 h with the various dsRNA sequences (50 nm final concentration) using Lipo-
concentration) using Oligofectamine (3 μl/well) reagent in 1 ml of OptiMEM medium (both from Invitrogen). 4 h after transfection, 500 μl of DMEM or McCoy’s medium containing 30% FCS, 300 units/ml penicillin, and 300 μg/ml streptomycin were added to each well. For double transfection experiments, HeLa cells were loaded twice with the dsRNA sequences, were seeded on gelatin-coated coverslips (Sigma and Fisher Scientific, respectively) in 24-well plates at a density of 2 × 10^4 cells/well. Cells were transfected after 2 days using Oligofectamine (0.75 μl/well) in Opti-MEM (250 μl). 4 h after transfection, 125 μl of DMEM or McCoy’s medium containing 30% FCS, 300 units/ml penicillin, and 300 μg/ml streptomycin was added and observed for co-sedimentation experiments. HeLa cells were then transfected with control GFP or GFP-survivin (pEGFP, Clontech) cDNAs using LipofectAMINE (Invitrogen) as previously described (22).

Western Blot and Subcellular Fractionation—Cells transfected with the various dsRNA sequences were collected, centrifuged at 3000 rpm for 5 min at 4°C, and washed in PBS, pH 7.2. The cell pellet was solubilized in lysis buffer (20 mM Tris, pH 7.2, 0.5% deoxycholate, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 1 mM EDTA) containing protease inhibitors (Complete, Roche Applied Science) for 30 min at 4°C. The lysate was cleared by centrifugation for 15 min, and protein concentration was evaluated using BCA assay (Pierce). Samples (20 μg) were separated by SDS-polyacrylamide gel, transferred to nylon membranes, and incubated with the following primary antibodies to survivin (Novus Biologicals), XIAP (BD Transduction Laboratories), β-actin (Sigma), caspase-9 (Cell Signaling Technology), Bcl-2 (BD Transduction Laboratories), p53 (Oncogene Research Products), p21 (Oncogene), GFP (Clontech), or proliferating cell nuclear antigen (PCNA, Sigma). Binding of the primary antibodies was determined by addition of horseradish peroxidase-conjugated secondary reagents, and bands were visualized by enhanced chemiluminescence (Amerham Biosciences). Subcellular fractionation experiments with isolation of individual nuclear or cytosolic fractions (20 μg) in RNAi-treated cultures were carried and characterized as described previously (18) and analyzed for changes in survivin expression by Western blotting.

Survivin-Microtubule Interaction—Co-sedimentation experiments were carried out as described previously (23). Briefly, untreated HeLa cells or HeLa cells transfected with control GFP or GFP-survivin cDNAs were suspended in MES buffer (0.1 M MES, 1 mM EDTA, 1 mM MgSO_4, pH 6.6) and lysed by multiple strokes through a 27-gauge needle. The extract was preclarified by centrifugation at 12,000 rpm for 5 min at 4°C, and the supernatant was further spun at 164,000 × g for 1 h at 4°C. The resulting cytosolic fraction was dialyzed in MES buffer for 3 h at 4°C, and aliquots of the extract (200 μl at 4 mg/ml) were used for microtubule assembly studies. Microtubules were polymerized in vitro in a taxol-free MES buffer by four sequential cycles of polymerization for 30 min at 37°C, followed by depolymerization for 30 min at 4°C. Samples were loaded on a 10% sucrose cushion in MES buffer and centrifuged at 30,000 × g for 30 min at 30°C. Supernatants (50% of the incubation reaction) and pellets (polymerized microtubules) were analyzed for the presence of co-associating survivin by Western blotting. Samples obtained from non-polymerized microtubules were loaded as negative controls.

Analysis of Apoptosis, Cell Cycle, and Mitochondrial Membrane Depolarization—Changes in apoptosis and cell cycle progression following interference with survivin expression were assessed by DNA content evaluation by propidium iodide staining and flow cytometry as previously described (22). The peaks associated with apoptotic (hypodiploid, sub-G1) and G2/M (4N), and multinucleated (>4N) cells were quantified using cell cycle data analysis software (FlowJo). For enhancement of apoptosis-inducing stimuli by survivin knockdown, HeLa cells were transfected with VIII or S4 dsRNA sequences, incubated for 36 h, and exposed to UVB irradiation at 100 J/m². After an additional 16 h culture at 37°C, HeLa cells were stained with a fluorescein-conjugated caspase-9 inhibitor (Z-VAD-fmk) and propidium iodide (CaspaTag, Intergen) for simultaneous detection of caspase activity and loss of plasma membrane integrity. The dot blots under the various conditions tested were analyzed using FlowJo flow cytometry software. In separate experiments, HeLa cells transfected with VIII or S4 dsRNA sequences and exposed to UVB were analyzed for localization of caspase-9 by Western blotting. Chinese hamster ovary cells transfected with pCF-Ul, a mitochondrion membrane potential in RNAi-transfected HeLa cells were monitored using the membrane potential-sensitive fluorescent dye JC-1 and flow cytometry. Briefly, HeLa cells were doubly transfected with control VIII or survivin-specific S4 dsRNA sequences, harvested after 24 h, and incubated for 10 min in the dark with JC-1 (10 μg/ml in PBS, Molecular Probes). After washing in PBS, pH 7.2, samples were analyzed by flow cytometry to monitor decreases in the red/green (FL-2/FL-1) fluorescence ratio (index of mitochondrial membrane depolarization). Cultures treated with staurosporine (3 μM, Sigma) for 24 h and stained with JC-1 served as positive control for these experiments.

Immunofluorescence Microscopy—HeLa or HCT-116 cells grown on gelatin-coated coverslips were fixed in paraformaldehyde (4% paraformaldehyde, 0.25% triton in PBS) for 20 min and permeabilized in cold (−20°C) methanol for 10 min. Cells were incubated for 10 min in PBS/glycine 20 mM and for 20 min in PBS/BSA 3% and further incubated with an antibody to β-tubulin (Sigma) for microtubule staining or an antibody to γ-tubulin (Sigma) for centrosomal staining. For localization of survivin to different aspects of the mitotic apparatus, HeLa cells were fixed in microtubule-stabilizing buffer as described in details in our previously published protocols (18). Coverslips were simultaneously incubated with a polyclonal antibody detecting the various subcellular pools of survivin (Novus) as described previously (18) and with an antibody to β-tubulin. After washes, binding of the primary antibodies was detected using fluorescein isothiocyanate- or rhodamine (Zymed Laboratories Inc.)-conjugated secondary antibodies of appropriate specificities. To reveal chromatin, following incubation with the secondary antibody, cells were washed twice in PBS, pH 7.2, and once in water, and stained for 5 min with DAPI (Sigma, 0.5 μg/ml in water). Coverslips were then mounted on glass slides (Fisher) and observed using a fluorescence inverted microscope (Olympus, IX71). Pictures were taken using a fluorescence microscope (Nikon) connected to a PC-compatible computer through a camera.

Statistical Analysis—Data were analyzed using the unpaired t test on a Graphpad software package for Windows (Prism). A p value of 0.05 was considered as statistically significant.

RESULTS
Acute Ablation of Survivin by RNAi Induces Mitotic Arrest and Polyplody—Preliminary experiments analyzed two survivin-derived dsRNA sequences for their ability to suppress survivin expression in model cervical carcinoma HeLa cells. Transfection of HeLa cells with survivin-specific S1 or S4 dsRNA oligonucleotides resulted in reduction in survivin levels, by Western blotting of whole cell lysates (Fig. 1A). In combination, expression of other anti-apoptotic regulators, XIAP or Bcl-2 was unaffected (Fig. 1A). Similarly, transfection of a control unrelated dsRNA sequence (VIII) did not reduce the expression of Bcl-2, XIAP or survivin in HeLa cells, by Western blotting (Fig. 1A). By 72 h after transfection, endogenous survivin levels had largely recovered in S1-transfected cultures, whereas transfection with the S4 sequence resulted in a more prominent and sustained suppression of survivin at 48 and 72 h (Fig. 1A). Cell cycle analysis revealed that transfection of HeLa cells with the S4, but not the VIII sequence resulted in a significant increase in the G2/M fraction, suggestive of cell cycle arrest, and appearance of polyplloid cells with >4N DNA content (Fig. 1, B and C), in agreement with recent observations (21, 24). When analyzed by fluorescence microscopy, S4-transfected cultures contained large, multinucleated cells, as compared with VIII-transfectants (54.4 ± 7.2% versus 1.1 ± 0.8%, n = 4) (Fig. 1D). Consistent with its reduced ability to affect survivin levels in whole extracts (Fig. 1A), the survivin S1 sequence (21) produced only a modest G2/M arrest, and negligible polyplody (Fig. 1, B and C).

Loss of Survivin Induces Multiple Mitotic Defects—The nature of the cell division defect induced by RNAi suppression of survivin was next investigated. Transfection of HeLa cells with the S4 sequence revealed a striking increase in the number of multipolar mitoses, as compared with VIII-transfected cultures (50 ± 7% versus 4 ± 7%, n = 4) (Fig. 2A). With respect to the kinetics of appearance of multipolar spindle defects and multinucleation, 45% of S4-treated HeLa cells exhibited spindle defects as opposed to 20% of the population with multiple nuclei 36 h after transfection. By 48 h, the number of multinucleated cells had increased to 54%, whereas the percentage of...
cells with multipolar spindles increased only slightly (50%). In addition, HeLa cell transfection with S4 resulted in profound defects in chromosome congression (Fig. 2A), with cell cycle arrest at prometaphase and reduction/loss of all subsequent mitotic phases, whereas VIII-transfected cultures had normal distribution of mitotic phases, by fluorescence microscopy (not shown).

The potential effect of RNAi targeting of survivin on centrosome number/organization (16, 22) was next investigated. By immunofluorescence staining with an antibody to centrosome-associated γ-tubulin, suppression of survivin by the S4 sequence resulted in a host of centrosomal defects, including appearance of supernumerary centrosomes, aberrantly small centrosomes and centrosomal fragments (Fig. 2, B and C). In contrast, no centrosomal defects were observed in cells transfected with the VIII sequence, by fluorescence microscopy (Fig. 2, B and C). Centrosome duplication is coordinated by a kinase cascade contributed by Cdk2-cyclin E (25). We investigated the effect of homozygous deletion of the cyclin-dependent kinase antagonist and p53 transcriptional target, p21Waf1/Cip1, once centrosomal deregulation induced by RNAi suppression of survivin. Transfection of HCT-116 p21+/−/p21−/− cells with the S4 sequence suppressed endogenous survivin levels, as compared with control VIII sequence, by Western blotting (Fig. 3A). Transfection of p21+/− or p21−/− cells with the VIII sequence did not affect mitotic spindle formation, and metaphase cells exhibited normal, bipolar mitotic spindles with full complement of microtubules and proper chromosome alignment, by fluorescence microscopy (Fig. 3, B and C). In contrast, transfection of the S4 sequence in p21−/− cells severely exacerbated the multiple cell division defects associated with survivin loss and resulted in the appearance of shortened, flattened spindles severely depleted of microtubules, monopolar, and multipolar spindles (Fig. 3, C and D), and abnormalities of chromosome congression, by DAPI staining (Fig. 3C).

Coupling of the Survivin Mitotic Checkpoint to p53—Because loss of the p53 transcriptional target, p21, exaggerated the mitotic defects associated with survivin suppression, we next tested a potential link between survivin and p53. Transfection of p53+/− or p53−/− HCT-116 cells with survivin dsRNA sequences, but not the control VIII sequence, resulted in a partial (S1) or complete (S4) ablation of survivin expression, by Western blotting of whole cell extracts (not shown). Under these experimental conditions, RNAi targeting of survivin resulted in time-dependent increased expression of p53 by Western blotting, compared with VIII-transfected cells (Fig. 4A). Double transfection of HCT-116 cells with S1 or S4 sequences at a 48-h interval resulted in maximal p53 induction (Fig. 4A). Induction of p53 after partial (S1) or complete (S4) survivin ablation was associated with increased expression of its transcriptional tar-

---

**Fig. 1.** Down-regulation of survivin by RNAi affects mitotic progression in HeLa cells. **A,** Western blotting. HeLa cells were transfected with a control (VIII) or two survivin-targeted dsRNA sequences (S1 and S4), harvested after a 48–72 h culture at 37 °C, and analyzed for expression of survivin, Bcl-2, XIAP, or β-actin (as a loading control) by Western blotting. *, nonspecific band. **B,** Cell cycle profile. HeLa cells transfected with the indicated dsRNA sequences were harvested after 48 h and analyzed for cell cycle progression by propidium iodide staining and flow cytometry. **C,** summary of cell cycle defects in survivin RNAi-treated HeLa cells. The experimental conditions are as in **B.** The percentage of cells with hypodiploid, 4N (G2/M) or >4N DNA content is indicated per each condition tested. Data are the mean ± S.E. of four independent determinations. **D,** fluorescence microscopy. HeLa cells transfected with control (VIII) or S4 dsRNA oligonucleotide were harvested after 48 h and stained with an antibody to β-tubulin (red). DNA was stained with DAPI (blue), and cells were analyzed by fluorescence microscopy. Arrows, multinucleated cells.
get, p21\textsuperscript{Waf1/Cip1}, by Western blotting (Fig. 4A). When analyzed for cell cycle defects, transfection of the S4 sequence in p53\textsuperscript{+/+} cells resulted in a sustained G2/M arrest and appearance of a small population with 4N DNA content (Fig. 4B). In contrast, the G2/M arrest induced by survivin ablation was lost in p53\textsuperscript{−/−} cells (Fig. 4B). In addition, p53\textsuperscript{−/−} cells continued to progress through mitosis despite the absence of survivin as revealed by the appearance of polyploid populations with >4N and >8N DNA content, as compared with S4-transfected p53\textsuperscript{+/+} cells (Fig. 4B). Consistent with the data presented in Fig. 1B, transfection of the S1 sequence was minimally effective at inducing G2/M arrest in p53\textsuperscript{+/+} HCT-116 cells (Fig. 4B).

**Survivin-Microtubule Interaction, in Vivo**—The possibility that the p53-regulated mitotic checkpoint functions of survivin...
could reflect its direct association with the mitotic apparatus was next investigated. A polyclonal antibody that recognizes all the various subcellular pools of survivin (18) was used for immunofluorescence microscopy (Fig. 5A). The same antibody to survivin also labeled the pool of survivin co-sedimenting with microtubules assembled by temperature-dependent polymerization, as compared with non-polymerized microtubules (Fig. 5B).

**Fig. 4. The mitotic checkpoint function of survivin requires p53.** A, p53 induction. HCT-116 p53+/− cells were transfected with control (VIII) or survivin-specific (S1 or S4) dsRNA sequences, harvested at the indicated increasing time intervals after transfection and analyzed for expression of p53, p21, or β-actin by Western blotting. In some experiments, cultures were transfected twice at 48-h intervals and harvested 24 h after the second transfection for Western blotting analysis (48 + 24). p53 bands in S1- or S4-treated cells were quantified by densitometry and normalized to β-actin content as -fold increase compared with VIII-transfected cultures. B, cell cycle analysis. Aliquots of p53+/− or p53−/− HCT-116 cells doubly transfected with control (VIII) or survivin-specific S1 or S4 dsRNA sequences were analyzed for changes in cell cycle progression by DNA content and flow cytometry. The percentage of cells in the various cell cycle phases is indicated. The cell cycle analysis was carried out after electronically gating out the fraction of apoptotic cells (sub-G1).

**DISCUSSION**

In this study, we have positioned survivin in a p53-dependent pathway (3) that surveys the progression through multiple phases of mitosis, and we have linked its second function in cytoprotection to the upstream regulation of mitochondrial-dependent apoptosis (26). These combined features fulfill the definition of survivin as a mitotic checkpoint required for genomic integrity and cytoprotection.

The first conclusion that can be drawn from these studies is that a reduction in the nuclear pool of survivin (18) was sufficient to cause a complete deregulation of cell division, in which mitotic arrest and polyploidy was one but several defects involving centrosome replication, microtubule nucleation, and mitotic spindle assembly/stability. This phenotype was exaggerated in cells lacking the Cdk inhibitor, p21Waf1/Cip1, suggesting that unbridled Cdk activity, most notably Cdk2/4/6, cooperates with loss of survivin in deregulating the proper...
assembly of a bipolar mitotic apparatus. These data agree well with earlier results obtained with survivin antisense oligonucleotides (22), and are consistent with the role of p21 and Cdk2-cyclin E in centrosome replication and microtubule nucleation in late S phase and early mitosis (25, 27), and the localization of survivin to centrosomes (16, 22). The dynamic association of survivin with other aspects of the mitotic apparatus, including microtubules of the metaphase and anaphase spindle, and kinetochores of metaphase chromosomes (18), may explain the multiple cell division defects of aberrant spindle

**FIG. 5.** Survivin-tubulin interaction. A–C, fluorescence microscopy. HeLa cells were fixed in microtubule stabilizing buffer and simultaneously stained with a polyclonal antibody to survivin (Novus, Texas Red) and antibody to β-tubulin (fluorescein isothiocyanate). DNA was stained with DAPI, and cells were analyzed by fluorescence microscopy for localization of survivin at metaphase (A), anaphase (B), or telophase (C). Image merging analysis is shown for the various conditions tested. Arrows indicate the localization of survivin to spindle microtubules and kinetochores at metaphase (A), central spindle midzone at anaphase (B), and midbodies at telophase (C). D, association of endogenous survivin with polymerized microtubules of HeLa cells. Microtubules were left unpolymerized by incubation at 0 °C or polymerized by four sequential cycles of incubation at 37 °C then 0 °C of 30 min each, centrifuged through a sucrose cushion and associated endogenous survivin in pellets or supernatants was detected by Western blotting. E, microtubule-association of GFP-survivin overexpressed in HeLa cells. The experimental procedures are as in D, except that HeLa cells were transfected with control GFP or GFP-survivin cDNA, followed by three sequential cycles of microtubule polymerization/depolymerization and analysis of survivin-microtubule interaction with an antibody to survivin by Western blotting.

**FIG. 6.** Differential ablation of survivin pools by RNAi enhances cell death stimuli. A, Western blotting of subcellular fractions. HeLa cells were transfected with control (VIII) or survivin-specific (S4) dsRNA sequence, harvested after 48 h and fractionated in nuclear and cytoplasmic extracts. Expression of survivin in both fractions was determined by Western blotting. PCNA and β-actin were used as loading controls for nuclear and cytosolic fractions, respectively. B, enhancement of UVB-induced apoptosis. HeLa cells were transfected with control (VIII) or survivin-specific (S4) dsRNA sequence and treated with 100 J/m² UVB 36 h later. Cells were analyzed by multiparametric flow cytometry for caspase-3 or -7 activity (DEVDase activity, green fluorescence) and cell death by propidium iodide staining (red fluorescence) after an additional 16-h incubation at 37 °C. The percentage of cells expressing DEVDase activity and/or propidium iodide labeling is indicated per each condition tested. C, caspase-9 activation. The experimental conditions are as in B, except that HeLa cells transfected with dsRNA sequences and exposed to UVB (100 J/m²) were harvested after 16 h and analyzed for cleavage of caspase-9 by Western blotting. The positions of proform caspase-9 and active caspase-9 fragments of 35 and 37 kDa are indicated.
formation and chromosome congression resulting from survivin ablation. A potential association of survivin with metaphase spindle microtubules has been debated in the literature and described by some investigators (16, 18) but not by others (14, 28). Here, we obtained fresh evidence in agreement with our previous data (23) that a pool of survivin decorates metaphase spindle fibers and that survivin physically associates with polymerized tubulin, in vivo. Recent claims that this immunolocalization pattern is unique to a single antibody to survivin (21) are inconsistent with published evidence. GFP-survivin was previously shown to label metaphase spindle microtubules in the absence of antibody detection (17), and the spindle staining described here was obtained with a different antibody to survivin (pAb Novus), previously characterized for recognition of the different survivin isoforms, including microtubule-associated survivin (18). Altogether, these findings suggest that survivin acts at multiple phases of mitosis, not simply cytokinesis, as previously inferred from the phenotype of IAP-like molecules in model organisms (15, 29). This model is consistent with emerging evidence that molecules such as Aurora B (30) or TD-60 (31) that share, at least in part, a common localization with survivin on the mitotic apparatus (28) also participate in multiple phases of mitosis (32, 33), including spindle formation (31), and not solely cell cleavage as originally proposed (30, 34).

A novel aspect of the survivin mitotic checkpoint uncovered here was its dependence on p53 (9). Although it was previously demonstrated that survivin is one of the few known genes transcriptionally repressed by p53 (7, 8), here, loss of survivin resulted in up-regulation of the p53 downstream transcriptional target, p21WafI/Cip1 (17), which also participates in p53-dependent G1/M arrest (35), via increased p53 expression and/or activity. In addition, the mitotic block induced by survivin ablation was lost in both S4-doubly transfected p53−/− HCT-116 and HeLa cells,2 which carry functionally inactivated p53, whereas wild type p53 limited the expansion of polyploid cells in survivin-targeted cultures. It is established that p53 functions in cell cycle arrest at both the G1/S and G2/M transitions (36) and that this pathway is essential for genomic integrity (37). The data presented here suggest that survivin may function as a novel relay of cell division defects that activates p53-dependent responses in mitotically abnormal cells, thus limiting their progression to aneuploidy. This is consistent with the role of p53 in limiting DNA endoreduplication and aneuploidy after spindle damage (38) and suggests that p53-dependent repression of the survivin gene (7, 8) may further integrate this checkpoint to lower an anti-apoptotic threshold in mitotically arrested cells.

In addition to mitotic checkpoint, survivin was shown here to directly couple to the most upstream events in mitochrondial-dependent apoptosis. That survivin cytoprotection may diverge from the general function of other IAP proteins as downstream caspase inhibitors (5) is not surprising and in agreement with published data (13). In fact, recent studies have repeatedly pointed to the mitochondrial pathway of apoptosis (26) as the target for survivin cytoprotection, first by demonstrating that apoptosis induced by a survivin Thr34Ala dominant negative mutant (39, 40) is ablated in mouse embryonic fibroblasts deficient in apoptosis components, Apaf-1 or caspase-9 (41), and, second, by identifying a cofactor (hepatitis B X-interacting protein) that enables survivin to prevent recruitment of caspase-9 to the apoptosome (42). The data presented here fit well with these findings and define a novel role of survivin in the regulation of mitochondrial-dependent apoptosis, independently of p53. At present, we can only speculate as to the mechanistic links between survivin and mitochondrial apoptosis. This may involve modulation of an upstream caspase that perturbs mitochondrial function, i.e. caspase-2 (43), or, alternatively, a direct effect on mitochondria, as recently proposed for one of the alternatively spliced survivin isoforms (44). Our studies also appear to pinpoint the cytoprotective function of survivin to its cytosolic pool (18). Intriguingly, cytosolic, but not nuclear survivin has been shown to be phosphorylated on Thr34

---

2 E. Beltrami, unpublished observations.
by p34<sup>res</sup> (18), a modification recently identified as a requirement for survivin cytoprotection via binding to HBXIP (42).

Some of the results presented here, particularly the apoptotic phenotype and the multiple cell division defects, differ sharply from those of two recent studies that used RNAi to target survivin (21, 24). Several points are noteworthy that may explain the differences. First and foremost, neither previous study checked for a potential differential susceptibility of the individual survivin pools to RNAi ablation (21, 24). It should also be pointed out that the various survivin pools are immunochemically distinct and recognized by different antibodies in a mutually exclusive fashion (18). This suggests that in the absence of subcellular fractionation experiments, the use of a single commercial antibody may not adequately validate the efficiency of RNAi suppression of the entire cellular survivin content (24). Second, the RNAi sequence used by Carvalho et al. (21), which is identical to the S1 sequence used here, was largely ineffective at reducing survivin levels in our hands, and the time course of survivin suppression reported by Carvalho et al., i.e. up to 84 h (21), is inconsistent with the recovery of survivin levels observed here 72 h after transfection. Whereas loss/interference with survivin function has been invariably associated with apoptosis in the literature (10), the persistence of survivin labeling on spindle microtubules, as detected by monoclonal antibody 8E2 by Carvalho et al., is an excellent agreement with those of RNAi targeting of survivin in (17), and survivin knockout embryos had a complete loss of mitotic spindle formation (14). In contrast, our results are in agreement with those of RNAi targeting of survivin in tumors (45), which resulted in mitotic arrest, inhibition of colony formation, and strong increase in spontaneous apoptosis with suppression of tumor growth in mice (45).

In summary, we have demonstrated that survivin targeting is an effective means to activate p53-dependent responses, block cell proliferation, and induce apoptosis in tumor cells. The p53 independence of apoptosis resulting from survivin ablation reinforces the suitability of targeting survivin for novel cancer therapies (10), whose efficacy would not be affected by the p53 status of the tumor. Although the independent regulation of distinct survivin subcellular pools calls for caution in interpreting results of RNAi ablation (21, 24), the data presented here position the survivin pathway as a novel checkpoint mechanism at the interface between mitotic progression and apoptosis control.

Acknowledgment—We thank Dr. Bert Vogelstein for genetically engineered HCT-116 cells.