Advances in Brief

Regulation of Microtubule Stability and Mitotic Progression by Survivin[1]

Alessandra Giodini,[2] Marko J. Kallio,[2] Nathan R. Wall, Gary J. Gorbsky, Simona Tognin, Pier Carlo Marchisio, Marc Symons,[2] and Dario C. Altieri[2,3]

Boyer Center for Molecular Medicine, Department of Pathology, Yale University School of Medicine, New Haven, Connecticut 06536 [A. G., N. R. W., D. C. A.]; University of Oklahoma Health Sciences Center, Biomedical Research Center, Oklahoma City, Oklahoma 73104 [M. J. K., G. J. G.]; Vita-Salute University School of Medicine, San Raffaele Scientific Institute, Milano 20132, Italy [S. T., P. C. M.]; and Center for Oncology and Cell Biology, North Shore-Long Island Jewish Research Institute, Manhasset, New York 11030 [M. S.]

Abstract

Survivin is a member of the inhibitor of apoptosis (IAP) gene family, which has been implicated in both preservation of cell viability and regulation of mitosis in cancer cells. Here, we show that HeLa cells microinjected with a polyclonal antibody to survivin exhibited delayed progression in prometaphase (31.5 ± 6.9 min) and metaphase (126.8 ± 73.8 min), as compared with control injected cells (prometaphase, 21.5 ± 3.3 min; metaphase, 18.9 ± 4.5 min; $P < 0.01$). Cells injected with the antibody to survivin displayed short mitotic spindles severely depleted of microtubules and occasionally underwent apoptosis without exiting the mitotic block or thereafter. Forced expression of survivin in HeLa cells profoundly influenced microtubule dynamics with reduction of pole-to-pole distance at metaphase (8.57 ± 0.21 μm *versus* 10.58 ± 0.19 μm; $P < 0.0001$) and stabilization of microtubules against nocodazole-induced depolymerization *in vivo*. These data demonstrate that survivin functions at cell division to control microtubule stability and assembly of a normal mitotic spindle. This pathway may facilitate checkpoint evasion and promote resistance to chemotherapy in cancer.

Introduction

Members of the IAP[4] gene family have been implicated in two cellular functions, suppression of apoptotic cell death and regulation of cell division. Whereas several mammalian IAP proteins counteract apoptosis by interfering with processing and catalytic activity of caspases (1), IAP proteins in yeast and the nematode *Caenorhabditis elegans* have been implicated in mechanisms of late-stage cell division, *i.e.*, cytokinesis (2–4). Among mammalian IAP proteins, survivin may exhibit both functions of apoptosis inhibitor and regulator of cell division (5). Expressed in mitosis in a cell cycle-dependent manner and localized to multiple components of the mitotic apparatus and centrosomes (6, 7), survivin expression counteracts apoptosis *in vitro* and in transgenic animals (6). In addition, antisense suppression of survivin (8) or homozygous deletion of survivin in mice (9) results in a catastrophic defect of mitosis with multipolar spindles and aberrant tubulin assembly. The survivin pathway may be exploited in cancer where the *survivin* gene is dramatically overexpressed and associated with abbreviated survival, accelerated recurrences, and resistance to therapy (6). Although the antiapoptotic function of survivin may involve upstream modulation of the intrinsic (mitochondrial) apoptotic cascade (10, 11), the mechanism(s) by which survivin participates in cell division is only beginning to emerge. In addition, the subcellular localization of survivin has been debated and described as associated with microtubules and centrosomes, (12) or reminiscent of "chromosomal passenger proteins," molecules believed to participate in cytokinesis by interacting with kinetochores and transferring to the central spindle midzone at anaphase (9, 13, 14). Recent findings reconciled these discrepancies and demonstrated that survivin exists in immunochemically distinct pools with both localizations in mitotic cells (7). Here, we sought to determine the function of survivin at mitosis.

Materials and Methods

Antibodies. A rabbit pAb NOVUS (NOVUS Biologicals, Littleton, CO) to survivin was characterized recently (7). A mAb 32.1 selectively recognizing kinetochore-associated survivin was also characterized (7).

Transfection Experiments. HeLa cells at ~50% confluency were transiently transfected with a survivin cDNA fused to GFP (10) using the Fugene 6 reagent (Roche Diagnostics Corp., Indianapolis, IN).

Microinjection. HeLa cells were microinjected in late S-G$_2$ phase, 4–8 h after a 16-h thymidine release, as described (12). Synchronized cultures were microinjected in the cytoplasm with pAb NOVUS (0.6 mg/ml) or in the nucleus with mAb 32.1 (0.6 mg/ml) plus Texas Red-labeled dextran (20 mg/ml) in PBS (pH 7.4), using an Eppendorf semiautomated microinjector (5246 Transjector; Eppendorf, Hamburg, Germany). Microinjected cells were fixed, and microtubules were visualized by immunofluorescence. Images were collected using an IX70 Olympus inverted microscope equipped with ×40 (0.85 NA) and ×60 (1.4 NA) objectives and Inovision (Raleigh, NC) image analysis software.

For time lapse video microscopy, a Burleigh piezoelectric MIS-5000 Microinjection Manipulation System (Burleigh Instruments, Inc., Victor, NY) and Zeiss Axiovert 25 inverted microscope equipped with a dry ×40 objective (Carl Zeiss, Inc., Thornwood, NY) were used. HeLa cells were injected at different phases of cell division in groups of at least five cells/mitotic phase (late prophase, early prometaphase, metaphase, and anaphase). pAb NOVUS to survivin was introduced into the cells at a concentration of 1.0 mg/ml diluted in KCl-PO$_4$ microinjection buffer (0.1 M KCl, 1.7 mM NaCl, 8.0 mM Na$_2$HPO$_4$, and 1.5 mM KH$_2$PO$_4$). Control cells were injected with nonimmune rabbit IgG (8.0 mg/ml). Immediately after microinjection, the injection chamber was placed on the prewarmed microscope stage on a Nikon Diaphot inverted microscope equipped with a dry ×40 objective and a long working distance condensor (Nikon Inc., Rockville, MD). Thereafter, the cells were monitored and cinematographed using a Photometrics Sensys CCD camera (Roper Scientific, Trenton, NJ) and Metamorph Imaging System (Universal Imaging Corp., Downingtown, PA). Each cell was monitored either for the duration of mitosis or, in case of cell cycle delay, for a maximum of 260 min. The captured images were processed using Adobe Photoshop and Corel Draw software. Statistical analysis of duration of mitotic phases was performed using two-factor ANOVA with a 95% confidence range.

Immunofluorescence Microscopy. Microinjected HeLa cells were fixed in cold methanol and labeled with a pAb to tyrosinated tubulin or with mAb 2.1 to tubulin (Sigma). Nuclei were stained with DAPI. For immunofluorescence of transfected cells, HeLa cells were processed using the following two protocols. In a pre-extraction protocol, HeLa cells were treated with 0.5%

Triton X-100 in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, and 2 mM $MgCl_2$) for 3–5 min and fixed in 3% formaldehyde containing 0.2% Triton X-100 for 15 min. Cells were washed in MBST buffer containing 10 mM 4-morpholinepropanesulfonic acid, 150 mM NaCl, plus 0.05% Tween 20, pH 7.4, before DAPI staining. This protocol maximizes GFP labeling of kinetochore-associated survivin by diminishing the diffuse cytoplasmic and spindle pole/fiber survivin-GFP signal. In a second protocol of cofixation/extraction, transfected HeLa cells were simultaneously fixed and extracted in 3% formaldehyde in PHEM containing 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid for 15 min, followed by washes in MBST and DAPI staining. This protocol allows for optimal labeling of survivin associated with spindle fibers and spindle poles by reducing the cytoplasmic survivin-GFP signal that otherwise masks the spindle pole/fiber labeling. Cells were labeled with mAb YL1/2 against tyrosinated α-tubulin (1:1000; DAKO) followed by a Cy3-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) at 1:600 dilution. DNA was counterstained with DAPI. The pole-to-pole distance of 20 representative metaphase transfected or nontransfected HeLa cells was measured using a Zeiss Axioplan microscope equipped with ×63 (1.4 NA) objective, a Hamamatsu Orca CCD camera (Hamamatsu Photonics), and Metamorph image analysis software (Universal Imaging Corp.). Statistical analysis was carried out using a two-tailed unpaired t test (GraphPad Software, San Diego, CA).

Regulation of Apoptosis and Microtubule Stability *in Vivo*. A replication-deficient adenovirus encoding wild-type survivin (pAd-Survivin) was constructed using the pAdTrack-CMV and pAdEasy-1 vectors, as described (11). HeLa cells at 2×10^5 in a 24-well plate were infected with pAd-Survivin or control pAd-GFP at MOI of 50 for 8 h in 300 μl of complete medium. For determination of apoptosis, transduced HeLa cells were incubated with increasing concentrations of Taxol (2–10 μM), harvested after 48 h, and analyzed for DNA content by propidium iodide staining and flow cytometry (8). For microtubule stability, transduced cells (>95%) were treated with 1 or 10 μM nocodazole for 30 min at 37°C, fixed in cold methanol, and analyzed for tubulin staining with mAb 2.1. DNA was stained with DAPI.

Results

Survivin Controls Chromosome Congression and Mitotic Progression. In HeLa cells microinjected with nonimmune rabbit IgG ($n = 15$), the average duration of prometaphase (time from breakdown of the nuclear envelope to alignment of all chromosomes at the spindle equator) was 21.5 ± 3.3 min, and the average duration of metaphase (time from alignment of the last chromosome at the spindle equator to separation of the sister chromatids) was 18.9 ± 4.5 min (Fig. 1A). In contrast, HeLa cells microinjected with pAb NOVUS (7) exhibited delays in both prometaphase (31.5 ± 6.9 min) and metaphase (126.8 ± 73.8 min; $P < 0.01$; Fig. 1A). Twelve of 15 cells injected with pAb NOVUS spent at least 62 min at metaphase (Fig. 1, A–C; range, 62–231 min; the maximum duration of a monitoring session of arrested cells was 260 min). Three pAb NOVUS-injected cells progressed through mitosis without any apparent effect. Of 15 injected cells examined, 4 cells showed delayed onset of anaphase (duration, 62, 78, 142, and 174 min, respectively) and died by apoptosis without exiting mitosis ($n = 2$) or thereafter ($n = 2$; Fig. 1, B and D). For the cells that, albeit delayed, completed the metaphase-anaphase transition ($n = 5$), cytokinesis proceeded normally, and the time from anaphase onset to telophase was indistinguishable in HeLa cells injected with pAb NOVUS or IgG (Fig. 1A).

Role of Survivin in Spindle Formation. Microinjection of HeLa cells with pAb NOVUS resulted in the generation of shortened mitotic spindles with severely reduced spindle microtubule density (Fig. 2, a–d and a'–d'). This phenotype was observed in >80% of cells injected with pAb NOVUS and was reminiscent of microtubule depolymerization induced by low concentrations of nocodazole (Fig. 2, q–r). Consistent with the data of time-lapse videomicroscopy (Fig. 1), all microinjected cells that progressed through mitosis exhibited normal formation of midbodies and underwent cytokinesis that was
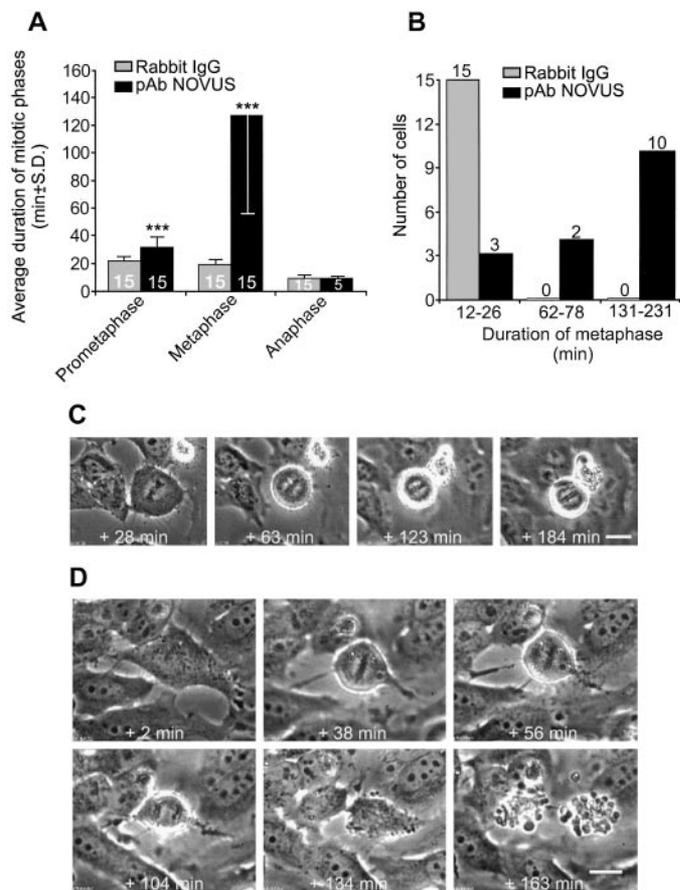

Fig. 1. Regulation of mitotic transitions by survivin. *A*, length of mitotic transitions in HeLa cells microinjected with rabbit IgG (8.0 mg/ml; $n = 15$) or pAb NOVUS to survivin (1.0 mg/ml; $n = 15$; $P < 0.01$). The number of cells in each category is indicated within the *column*. *B*, duration of metaphase in microinjected HeLa cells. All fifteen cells microinjected with IgG and three pAb NOVUS-injected cells progressed through metaphase without apparent delays (duration of metaphase ranged from 12 to 26 min). Two pAb NOVUS-injected cells exhibited a metaphase delay of 62 and 78 min, respectively, before anaphase and exit from M phase. These two cells underwent apoptosis in early G_1 (see *D*). Ten of 15 HeLa cells injected with pAb NOVUS remained arrested at metaphase (range, 131–231 min) for the entire duration of the monitoring session (180–260 min). Two of these cells died via apoptosis at metaphase after a metaphase delay of 142 and 174 min, respectively. The number of cells in each category is indicated above the *column*. *Bars: A* and *B*, SD. *C*, metaphase arrest in HeLa cells microinjected with pAb NOVUS. A HeLa cell was microinjected with pAb NOVUS at early prometaphase (nuclear envelope breakdown is time point 0 min). Approximately 30 min later, all of the chromosomes were aligned at the spindle equator. The cell arrested at metaphase for the entire duration of the monitoring session (~190 min). *D*, metaphase delay and apoptosis in HeLa cells microinjected with pAb NOVUS. A HeLa cell was injected with pAb NOVUS at late prophase (nuclear envelope breakdown is time point 0 min). Approximately 38 min after nuclear envelope breakdown, all of the chromosomes were aligned at the spindle equator. The sister chromatids separated after a metaphase delay of 62 min. The cell exited mitosis (~130 min from nuclear envelope breakdown) and died by apoptosis (~155 min from nuclear envelope breakdown). *Scale bars: C* and *D*, 10 μm.

indistinguishable from control-injected cells (Fig. 2, a''–d''; see below). HeLa cells microinjected with nonimmune rabbit IgG did not exhibit defects in mitotic spindle assembly (Fig. 2, e–h) or cytokinesis (not shown). Microinjection of synchronized HeLa cells in the nucleus with mAb 32.1 against kinetochore-associated survivin (Ref. 7; Fig. 2, i–l) or nonimmune mouse IgG (Fig. 2, m–p) did not result in defects of mitotic spindle assembly or cytokinesis.

Effect of Survivin on Spindle Dynamics. Pre-extraction of HeLa cells transfected with GFP-survivin resulted in intense GFP labeling of metaphase chromosomes and faint labeling of spindle poles (Fig. 3A). In contrast, simultaneous extraction/fixation of HeLa cells transfected with GFP-survivin resulted in strong GFP labeling of spindle poles and spindle microtubules (Fig. 3A). Expression of GFP-survivin resulted in significantly reduced pole-to-pole distance in
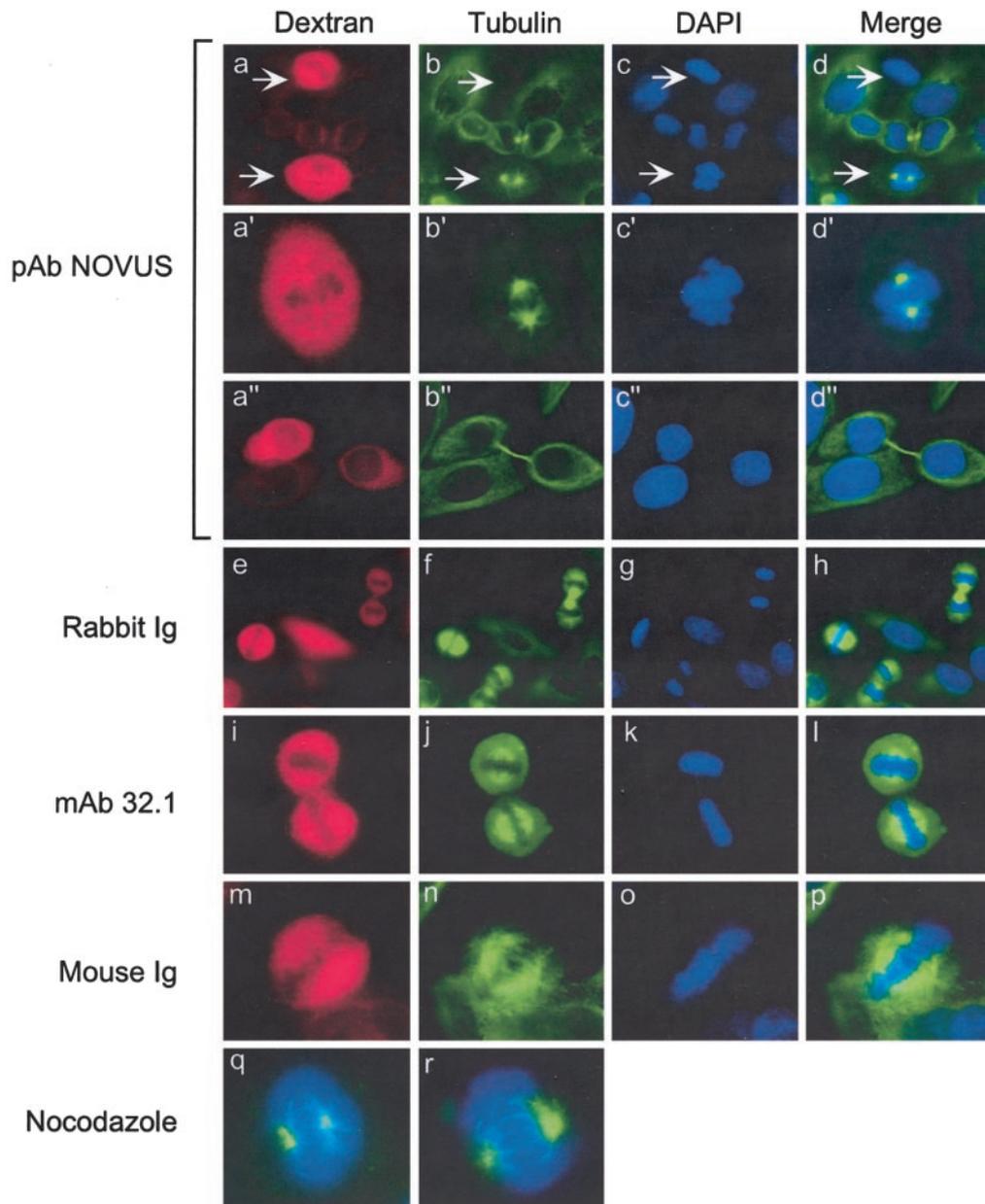
Fig. 2. Antibody targeting of survivin results in short mitotic spindles depleted of microtubules. Synchronized HeLa cells were microinjected with pAb NOVUS (*a–d, a'–d',* and *a"–d"*) or mAb 32.1 (*i–l*) to survivin or control nonimmune rabbit (*e–h*) or mouse (*m–p*) IgG 4–6 h after thymidine release. Cells were fixed, labeled with an antibody to tubulin, followed by a FITC-conjugated secondary antibody, and analyzed by fluorescence microscopy. Microinjected cells are identified by Texas Red dextran (*red* fluorescence). DNA was counterstained with DAPI. *q* and *r,* synchronized HeLa cells were treated with 5 nM nocodazole for 30 min and stained for microtubules (tubulin) and DNA (DAPI).

metaphase cells, as compared with nonexpressing HeLa cells on the same coverslip (Fig. 3, *B* and *C*). The average length of the metaphase spindle in GFP-survivin transfectants was 8.57 ± 0.21 μm (range, 6.72–10 μm) as opposed to 10.58 ± 0.19 μm (range, 9.4–12.67 μm) in nonexpressing cells (*P* < 0.0001; *n* = 20; Fig. 3*C*). A similar reduction in spindle length was also observed in prometaphase cells expressing GFP-survivin, as compared with nontransfected cells (not shown).

Survivin Stabilizes Microtubules *in Vivo*. Transduction of HeLa cells with pAd-Survivin resulted in prominent expression of a M_r ~16,500 survivin band, by Western blotting (Fig. 4*A*). In contrast, pAd-Survivin did not affect the endogenous levels of another anti-apoptotic IAP family member, XIAP, and conversely, pAd-GFP did not modulate the expression of XIAP or survivin in HeLa cells (Fig. 4*A*). Infection of HeLa cells with pAd-Survivin suppressed apoptosis induced by increasing concentrations (1–10 μM) of Taxol (Fig. 4*B*), in agreement with previous observations (12). Under these experimental conditions, HeLa cells infected with pAd-GFP and treated with nocodazole rounded up and exhibited dramatic disruption of interphase microtubules (Fig. 4*C*). In contrast, infection of HeLa cells with pAd-Survivin completely protected microtubules from nocodazole-induced depolymerization and preserved the integrity of interphase microtubules indistinguishably from untreated cultures (Fig. 4*C*).

Discussion

In this study, we have shown that microinjection of an antibody recognizing all of the immunochemically distinct mitotic pools of survivin (7) resulted in delayed prometaphase and metaphase stages and formation of aberrantly shortened mitotic spindles severely depleted of microtubules. In addition, overexpression of survivin using plasmid transfection or adenoviral transduction influenced microtubule dynamics with reduction in pole-to-pole distance at metaphase and stabilization of microtubules *in vivo*.

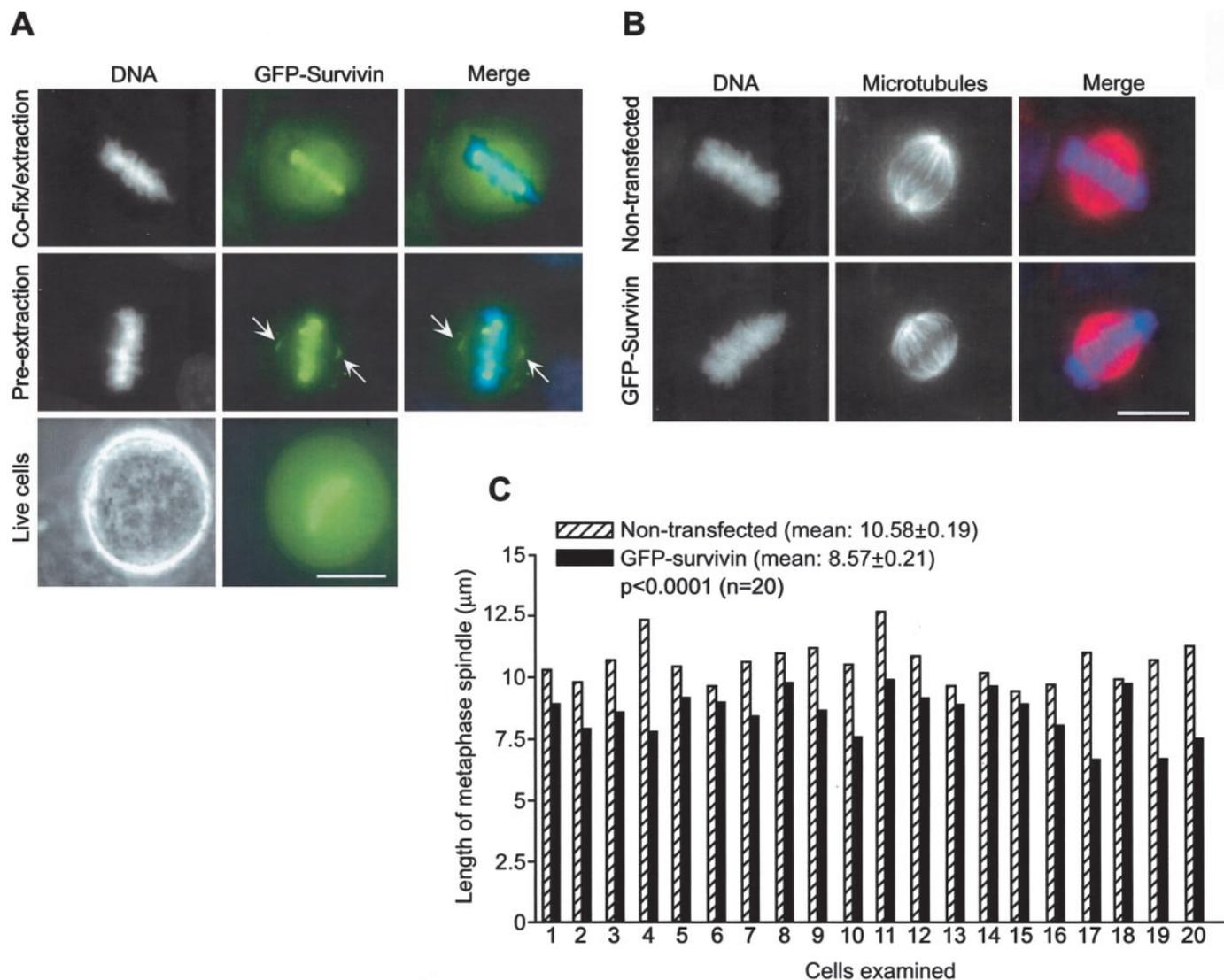Survivin may influence microtubule dynamics and promote increased

Fig. 3. Effect of GFP-survivin on spindle microtubule length. *A,* localization of GFP-survivin to centromeres, spindle poles, and spindle fibers. HeLa cells were transfected with GFP-survivin and either pre-extracted with 0.5% Triton X-100 before fixation in 3% paraformaldehyde in PHEM buffer (*Pre-extraction*), simultaneously fixed and extracted (*Co-fix/extraction*) in 3% paraformaldehyde in PEHM buffer containing 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid, or analyzed for GFP expression before extraction/fixation (*Live cells*). *Arrows,* GFP expression of survivin to spindle poles in pre-extracted HeLa cells. DNA was stained with DAPI. *B,* morphology of mitotic spindles. Nontransfected HeLa cells (*Non-transfected*) or transfected with GFP-survivin (*GFP-Survivin*) were analyzed at metaphase by fluorescence microscopy with an antibody to tyrosinated tubulin (*Microtubules*). DNA was stained with DAPI. *Right,* image merging analysis. *Scale bar,* 10 μm. *C,* interpolar distance. The pole-to-pole distance of 20 individual metaphase cells was measured by fluorescence microscopy with an antibody to tyrosinated tubulin and quantified using a CCD camera. The mean microtubule lengths of nontransfected cells or HeLa cells transfected with GFP-survivin are shown.

microtubule stability by directly regulating growth/catastrophe rates or via recruitment of MAPs (15) or motor proteins participating in spindle dynamics (16). Survivin exhibits several features found in MAPs, including a charged COOH terminus α-helix containing a tubulin binding site(s) (12), a physical association with polymerized tubulin regulated by microtubule dynamics (12), and a conserved p34[cdc2] phosphorylation site on Thr-34 that in other MAPs controls the affinity of tubulin recognition (15). The shortened spindle microtubules observed in GFP-survivin-expressing cells is also consistent with a stabilizing role of survivin on microtubule dynamics. In this context, stabilization of microtubules by Taxol (17) was associated with shortened metaphase microtubules attributable to removal of kinetochore-microtubule subunits from the centrosome in the absence of plus-end assembly (18).

A role of survivin at cell division via regulation of spindle dynamics is consistent with its localization to centrosomes, spindle poles and spindle microtubules (7, 12), and with the catastrophic loss of mitotic spindle formation of survivin knockout mice (9). In addition, a role of

survivin in spindle function was proposed in a recent study in which microinjection of an antibody to the COOH terminus of survivin, and thus different from the reagent used here, revealed premature entry into anaphase of the targeted cells, potentially reflecting modulation of spindle checkpoint signaling (19).

At variance with Wheatley *et al.* (14), who localized GFP-survivin exclusively to kinetochores, we observed prominent labeling of spindle poles and spindle microtubules in cells transfected with GFP-survivin. As shown here, this may be explained by differences in experimental protocols of fixation/extraction. Similar technical differences in preserving microtubule integrity may have also accounted for the failure of Wheatley *et al.* (14) to localize endogenous survivin to spindle poles using pAb NOVUS (7).

Despite the localization of a subcellular pool of survivin to the anaphase central spindle (7, 9, 13, 14) and the similarity with a nematode IAP protein (3, 4), the functional data presented here and in two preceding studies (7, 19) argue against a primary role of

Fig. 4. Survivin stabilizes microtubules *in vivo*. *A,* adenoviral expression of survivin. HeLa cells were infected with a replication-deficient adenovirus encoding wild-type survivin (*pAd-Survivin*) or control GFP (*pAd-GFP*) at a MOI of 50, harvested after 48 h, and analyzed by Western blotting with antibodies to survivin, XIAP, or β-tubulin. *B,* apoptosis inhibition by expression of pAd-Survivin. HeLa cells were infected with pAd-Survivin or pAd-GFP at a MOI of 50 and incubated with the indicated concentrations of Taxol for 48 h at 37°C. Apoptosis was determined from the fraction of cells with hypodiploid (sub-G$_1$) DNA content by propidium iodide staining and flow cytometry. Apoptosis in Taxol-treated cells in the absence of adenoviral transduction was 9.95 ± 1.25 (2 μM) and 21.1 ± 1.1 (10 μM). Data are the means of three independent experiments; *bars,* SE. *C,* stabilization of microtubules *in vivo*. Nontransduced HeLa cells (*Untreated*) were incubated in the absence (*None*) or the presence (*Nocodazole*; 5 μM) of the microtubule-depolymerizing drug, nocodazole. After infection with pAd-GFP or pAd-Survivin, cells were incubated with the indicated increasing concentrations of nocodazole for 30 min at 37°C, labeled with an antibody to tubulin (red), and analyzed by fluorescence microscopy. DNA was stained with DAPI.
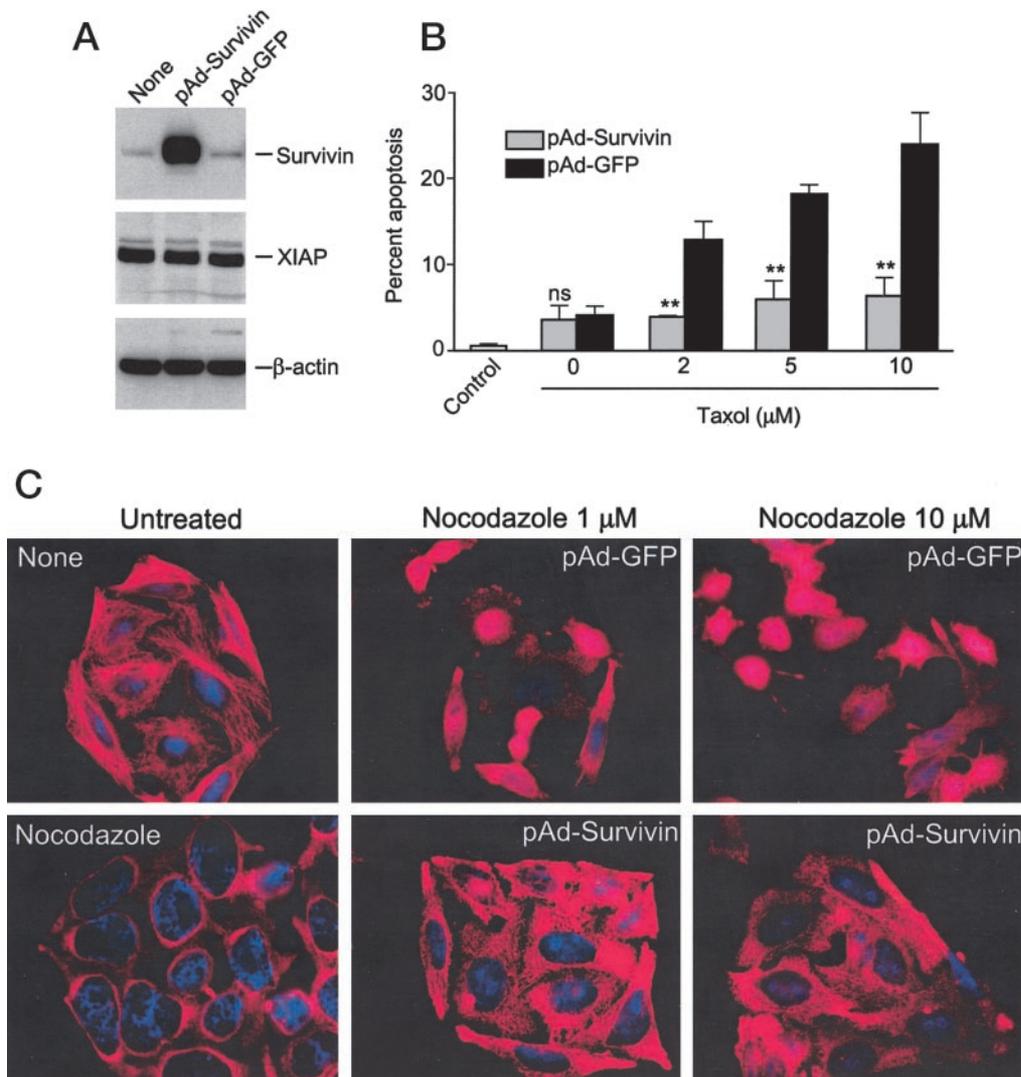
survivin in cytokinesis. At variance with the phenotype of IAP ablation in *C. elegans* (3, 4), antibody targeting of survivin caused apoptosis either coinciding with the sustained metaphase block or immediately thereafter. Altogether, these data suggest that survivin and IAP proteins in yeast (2) and *C. elegans* (3, 4) are evolutionary divergent in their roles at cell division, and that the proposed definition of survivin as a "chromosomal passenger protein" (13, 14) is inconsistent with its localization to metaphase spindle fibers (7, 12, 20) and its function on spindle dynamics (this work).

The data presented here may have critical implications for mechanisms of chemoresistance in cancer, where the *survivin* gene is dramatically overexpressed (6). Accordingly, survivin counteracts apoptosis induced by various chemotherapeutic drugs, including Taxol (12), and its expression in cancer correlates with chemoresistance *in vivo* (6). Other modulators of microtubule dynamics, including stathmin/Op18, are also exploited during tumorigenesis and become overexpressed in cancer (15). In this context, a dual role of survivin in apoptosis inhibition and regulation of spindle dynamics may facilitate evasion from checkpoint mechanisms of growth arrest and promote resistance to chemotherapeutic regimens targeting the mitotic spindle.

Acknowledgments

Note Added in Proof

Consistent with the findings presented here, Tran *et al.* have recently independently shown that survivin is a critical mediator of resistance to chemotherapy and preserves microtubule integrity in entothelial cells (Tran *et al.*, Proc. Natl. Acad. Sci. USA, *99:* 4349–4354, 2002).

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