Nuclear export is essential for the tumor-promoting activity of survivin

Shirley K. Knauer,* Oliver H. Krämer,† Thomas Knösel,‡ Knut Engels,§ Franz Rödel,‖ Adorján F. Kovács,¶ Wolfgang Dietmaier, Ludger Klein-Hitpass,†† Negusse Habtemichael,* Andrea Schweitzer,* Jürgen Brieger,‡‡ Claus Rödel,‖ Wolf Mann,†† Iver Petersen,‡ Thorsten Heinzel,† and Roland H. Stauber*,[1]**

*Georg-Speyer-Haus, Institute for Biomedical Research, Frankfurt, Germany; †Institute of Biochemistry and Biophysics, University of Jena, Jena, Germany; ‡Institute of Pathology, Charité-CCM, Medical Faculty Humboldt-University Berlin, Berlin, Germany; §Department of Pathology, University Hospital of Frankfurt, Frankfurt, Germany; ‖Department of Radiation Oncology, University of Erlangen-Nuremberg, Erlangen, Germany; ¶Maxillofacial Plastic Surgery, University Hospital of Frankfurt, Germany; **Institute of Pathology, University of Regensburg, Regensburg, Germany; ††Institute for Cell Biology, University Hospital of Essen, Essen, Germany; and ‡‡Department of Otorhinolaryngology, University Hospital of Mainz, Mainz, Germany

ABSTRACT **Survivin appears to function as an apoptosis inhibitor and a regulator of cell division during development and tumorigenesis. Here we report the molecular characterization of the nucleocytoplasmic transport of survivin and its potential implications for tumorigenesis. We identified an evolutionary conserved Crm1-dependent nuclear export signal (NES) in survivin. In dividing cells, the NES is essential for tethering survivin and the survivin/Aurora-B kinase complex to the mitotic machinery, which in turn appears to be essential for proper cell division. In addition, export seems to be required for the cytoprotective activity of survivin, as export-deficient survivin fails to protect tumor cells against chemo- and radiotherapy-induced apoptosis. These findings appear to be clinically relevant since preferential nuclear localization of survivin correlated with enhanced survival in colorectal cancer patients. Targeting survivin's nuclear export by the application of NES-specific antibodies promoted its nuclear accumulation and inhibited its cytoprotective function. We demonstrate that nuclear export is essential for the biological activity of survivin and promote the identification of molecular decoys to specifically interfere with survivin's nuclear export as potential anticancer therapeutics.—Knauer, S. K., Krämer, O. H., Knösel, T., Engels, K., Rödel, F., Kovács, A. F., Dietmaier, W., Klein-Hitpass, L., Habtemichael, N., Schweitzer, A., Brieger, J., Rödel, C., Mann, W., Petersen, I., Heinzel, T., Stauber, R. H. Nuclear export is essential for the tumor-promoting activity of survivin. *FASEB J.* 21, 207–216 (2007)**

Key Words: nucleocytoplasmic transport · apoptosis · Crm1 · head and neck cancer · colorectal cancer · cancer therapy · LMB · cisplatin · valproic acid · HDAC

An important advance in cancer research has been the finding that resistance to apoptosis is an important characteristic of human cancers (1, 2) which facilitates acquisition of additional cancer traits that promote resistance to therapy and disseminated disease. Among several mechanisms, escape from apoptosis can be the result of deregulated expression of apoptosis inhibitors (3). Considerable therapeutic and prognostic interest is focused on survivin (4), which at 16.5 kDa is the smallest mammalian member of the inhibitor of apoptosis protein (IAP) family (5). Survivin contains a single baculovirus IAP repeat and exists as a stable homodimer in solution (6). A single-copy survivin gene also gives rise to four alternatively spliced survivin transcripts (ref. 7 and references within). Survivin is largely undetectable in differentiated tissues but is highly expressed in most human tumors; its expression has been reported to correlate with reduced tumor cell apoptosis, increased resistance to cancer therapy, and abbreviated patient survival (4). However, the mechanims by which survivin counteracts apoptosis are still controversial (3, 4). Several reports suggest that survivin interacts directly with Smac/DIABLO (6), can form complexes with other IAP members (8), and binds to procaspases (9, 10) or Hsp90 (11). On the other hand, survivin has been defined as an essential component of the chromosomal passenger complex (CPC) (12). Among other factors, CPC includes the Aurora-B kinase and plays crucial roles during cell division (12, 13). Disturbance of CPC function by RNAi-mediated depletion of survivin or Aurora-B kinase has also been shown to induce apoptosis (see refs. 3, 12).

The diverse intracellular localization of survivin reported ranges from predominantly cytoplasmic, nu-

[1] Correspondence: Georg-Speyer-Haus Institute for Biomedical Research, Paul-Ehrlich-Str. 42–44, D-60596 Frankfurt, Germany. E-mail: stauber@em.uni-frankfurt.de

doi: 10.1096/fj.06-5741com

clear, and mitochondrial to components of the mitotic apparatus (4, 12). Although the low molecular weight would theoretically allow survivin to access intracellular compartments by passive diffusion, regulated subcellular localization has been proposed for survivin and other key players of apoptosis (see ref. 14). Nucleocytoplasmic transport is regulated by specific signals and transport receptors, and takes place through the nuclear pore complex (see ref. 15). Active nuclear import is mediated by short stretches of basic amino acids, termed nuclear localization signals, which interact with import receptors (15). The best-characterized nuclear export signals (NESs) are leucine-rich, interact with the export receptor Crm1, and depend on the RanGTP/GDP axis (15). Active transport signals have been identified in many cellular proteins executing crucial heterogeneous biological functions (see ref. 16). Thus, specific interference with regulated nucleocytoplasmic transport as a novel therapeutic principle has attracted great interest from academia and industry (reviewed in ref. 17).

To elucidate further survivin's physiological roles and to credential the survivin pathway for novel targeted interference approaches, we characterized the molecular regulation of survivin's dynamic localization. We show that mammalian survivin homologues are actively exported via a Crm1-dependent NES. The integrity of the NES in survivin is essential to guard tumor cells against cancer therapy-induced apoptosis and is required for proper cell division. Based on our results, we propose that survivin's nuclear export is biologically relevant and appears to be accessible to pharmacological interventions.

MATERIALS AND METHODS

Patient characteristics and biopsy samples

Tissue samples were obtained from patients at the Charité University Medical Center Berlin, the Department of Radiation Oncology University Erlangen-Nuremberg, the Department of Otolaryngology University Mainz, and the Department of Maxillofacial Plastic Surgery University Frankfurt. The study protocols were approved by the local ethics committees after obtaining the patients' informed consent to participate in the study; samples were processed anonymously. All cases were diagnosed histopathologically as colorectal carcinoma (CRC) or head and neck squamous carcinoma (HNSCC), and staged according to the TNM classification. The biopsy of macroscopically normal mucosa (NOM) was taken at a distance of >3 cm surrounding the tumor location. Tissue specimens were flash frozen in liquid nitrogen and stored until extraction of mRNA after removal of portions needed for pathological diagnosis. Histological analyses ensured that tumor specimen contained >70% tumor tissue and <10% necrotic debris, and samples not meeting these criteria were rejected. On average, tissues contained 40% epithelial mucosa and 60% submucosa tissue.

RNA extraction, reverse transcription, and quantitative real-time polymerase chain reaction analysis (RT-qPCR)

Total RNA was purified from patient material or cells using TRIZOL® reagent (Invitrogen Life Technologies, Karlsruhe, Germany) (18), and changes in mRNA levels were compared by reverse transcription (RT) and subsequent quantitative real-time polymerase chain reaction (qPCR) analysis as described (18). To define relative gene expression, the PCR product from each tumor sample was compared with the PCR product from NOM of the same patient. The relative expression ratio (R) of the target gene was calculated using the equation:

$$R = \frac{(E_{\text{target}})^{\Delta CP_{\text{target (control-sample)}}}}{(E_{\text{ref}})^{\Delta CP_{\text{ref (control-sample)}}}}$$

based on its real-time PCR efficiencies E, the crossing point (CP) difference of the tumor sample *vs.* NOM in comparison to the expression of the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (18). Primers (Invitrogen Life Technologies) were human *survivin wild-type* (*WT*), 5′-ATGGCCGAGGCTGGCTTCATC-3′ (sense) and 5′-GCGCAACCGGACGAATGCT-3′ (antisense); *survivinΔexon3*, 5′-ATGGCCGAGGCTGGCTTCATC-3′ (sense) and 5′-GCACTTTCTCCGCAGTTTCCTC-3′ (antisense).

Microarray expression analysis

Ten micrograms of total RNA was used to prepare biotinylated cRNAs. Hybridization of 15 μg labeled cRNA was performed on HG-U133A Affymetrix microarrays (Affymetrix, Santa Clara, CA, USA) as described (18). All arrays were globally scaled to a target value of 1000, and survivin expression intensity values were derived using Microarray Suite 5.0 as described (18).

Statistical analysis

Survival analysis was applied to all patients and was performed with SPSS® software (Munich, Germany) as described (19). Differences of the Kaplan-Meier survival curves were tested for statistical significance with the log-rank test, and the 95% confidence intervals were calculated. Differences were considered to be significant for $P < 0.05$.

Plasmids

Eukaryotic and bacterial expression constructs for green fluorescent protein (GFP) -tagged and untagged versions of human and rat survivin WT and mutants were constructed by PCR amplification using appropriate primers containing *Bam*HI/*Nhe*I-restriction sites. PCR products were cloned into the vector pc3-GFP or pGEX-GFP according to (20) (**Table 1**). To generate NES-deficient survivin, critical aa were changed by mutagenesis as described (20). Likewise, siRNA-resistant human survivin mutants were generated by introducing silent mutations at positions essential for siRNA binding (nucleotides ^{1229}C→ T and ^{1236}A→ C). Plasmids p3-Crm1-HA encoding hemagglutinin (HA) -tagged CRM1, pRev14-GFP encoding a cytoplasmic HIV-1 Rev mutant, p3AuroraB encoding Aurora-B kinase (21), and pDsRed encoding the red fluorescent protein (RFP) were reported (22).

Purification of recombinant glutathione S-transferase fusion proteins

Glutathione S-transferase-green flourescent protein (GST-GFP) hybrid proteins were expressed and purified from bacteria as described (20). GST-RanQ69L was purified in the presence of 0.1 mM GTP and stored in Ran-Buffer (50 mM HEPES, pH 7.6, 100 mM NaCl, 2 mM $MgCl_2$, 0.5 mM EDTA,

TABLE 1. *Analysis of the transport activity of WT and survivin mutants*[a]

Protein	Aa	Export activity		Import activity
		tf	mj	mj
Survivin	1–142	+(2, H, HN, CC)	+(V, HN, CC)	−(V, HN, CC)
survivin (rat)	1–142	+(2, H, HN, CC)	+(V, HN, CC)	−(V, HN, CC)
$survivin_{\Delta NES}$	1–142	−(2, H, HN, CC)	−(V, HN, CC)	−(V, HN, CC)
survivin__sim	1–142	+(2, H, HN, CC)	+(V, HN, CC)	−(V, HN, CC)
$survivin_{\Delta NES}$__sim	1–142	−(2, H, HN, CC)	−(V, HN, CC)	−(V, HN, CC)
$Survivin_{1–119}$	1–119	+(H, HN, CC)	n.d.	n.d.
$Survivin_{1–88}$	1–88	−(H, HN, CC)	n.d.	n.d.
survivinNES	89–98	n.d.	+(V, HN, CC)	n.d.
survivinNESmut	89–98	n.d.	−(V, HN, CC)	n.d.
$Survivin_{93–104}$	93–104	n.d.	−(V)	n.d.

[a]2, 293T; H, HeLa; HN, 1624; CC, RKO; V, Vero; N, NIH3T3; tf, transient expression; mj, microinjection; n.d., not done.

1 mM DTT, 0.1 mM GTP) after proteolytic removal of GST to ensure GTP loading.

Cells, transfection, microscopy, and microinjection

The CRC cell line RKO, the HNSCC cell line 1624, HeLa, Vero, and 293 cells were maintained under conditions recommended by the American Type Culture Collection (Manassas, VA, USA). Cells were prepared for microinjection or transfected as described (20). Microinjection of recombinant GST-GFP fusion proteins, observation, image analysis, and quantification of protein colocalization were performed as described in detail (20, 23). Laser scanning confocal microscopy was performed using a Leica TCS SL microscope (Leica Microsystems, Bensheim, Germany) as described (25). DNA/cell nuclei were visualized by staining with Hoechst 33258 (Sigma Aldrich, Munich, Germany) as described (20). Cell lines stably expressing survivin-GFP fusion proteins were selected with G418 (23).

Drug treatment and irradiation

Cells were treated with the export inhibitor leptomycin B (LMB) (10 nM), 1.5 mM valproic acid (VPA), 1.5 mM sodium butyrate, or 3 mM cisplatin (Sigma Aldrich) as described (20, 24). Radiation was delivered using a CIS IBL437 cesium irradiator (CIS, France) (dose rate=5.2 Gy/min with a single dose of 8 Gy). Sham-irradiated cultures were kept in the X-ray control room and the other samples were irradiated. After irradiation, the cells were kept in culture for 48 h.

Immunoprecipitation, immunoblotting, immunofluorescence, and immunohistochemistry (IHC)

Immunoblotting and immunofluorescence were carried out according to standard procedures (20). Immunohistochemical staining of survivin was performed using the polyclonal anti-(α)-survivin antibody (Ab) (Novus NB 500–201, Novus Biologicals, Littleton, CO, USA; CRC samples: 1:2000; HNSCC samples: 1:1000) and the FastRed chromogen® (CRC samples) (Immunotech, Hamburg, Germany) or the EnVision® detection system (HNSCC samples) (DakoCytomation GmbH, Hamburg, Germany) conjugated with horseradish peroxidase and 3,3 diaminobenzidine as the chromogen as described (19). The slides were finally counterstained with 50% hematoxylin and examined by light microscopy. The overall intracellular localization of survivin in the tumors was evaluated independently by two pathologists and scored as: −, negative; +C, predominantly cytoplasmic (>80% of survivin-positive tumor cells displayed cytoplasmic staining); +N, predominantly nuclear (>60% of survivin-positive tumor cells displayed nuclear staining). Before staining of patient material, the specificity of the α-survivin Ab and optimization of the staining protocol were performed on formalin fixed, paraffin-embedded RKO and 1624 cells expressing survivin-GFP.

The following antibodies were used in the study: α-survivin (Novus NB 500–201; Novus Biologicals, Littleton, CO, USA); anti-β-actin (A2066), anti-α-tubulin (T5168) and α-Aurora-B kinase (A5102) (Sigma Aldrich); α-caspase-3 (H-277) and α-caspase-9 (F-7) (Santa Cruz Biotechnology, Heidelberg, Germany). Appropriate Cy3- or FITC-conjugated secondary antibodies (Sigma Aldrich) were used.

Synthetic survivin NES peptide (^{89}VKKQFEELTLGEFLK^{103}) was derivatized with an amino-terminal cysteine residue, coupled to keyhole limpet hemocyanin, and used to immunize rabbits according to standard protocols (Pineda Ab Service, Hamburg, Germany) (20). The serum was preincubated with GST-SurvivinNESmut-GFP protein bound to glutathione Sepharose beads to remove unspecific binders, and subsequently affinity purified against the peptide using the SulfoLink coupling gel according to the manufacturer's instructions (Pierce Chemical Co., Rockford, IL, USA).

Crm1 pull-down assays and *in vitro* translation

Coupled transcription/translation was performed using the TNT reticulocyte lysate system (Promega, Heidelberg, Germany) supplemented with [^{35}S]-labeled methionine (Amersham Pharmacia Biotech, Buckinghamshire, UK) and the plasmids p3Crm1-HA, p3AuroraB-GFP, and p3-survivin-GFP as the templates. Pull-down assays with the specific recombinant GST-GFP substrates prebound to glutathione Sepharose beads were performed as described (20). Briefly, 4 μg of the bound GST-GFP proteins was incubated with equal amounts of *in vitro* translated Aurora B, survivin, or Crm1 (in the presence of 5 mM GTP-RanQ69L). After several washing steps, complexes were resolved by SDS-PAGE and visualized by fluorography (20).

RNAi

The sequence and activity of the survivin double-stranded siRNA (Eurogenetec, Searing, Belgium) (sense: 5′-CUGGA*C*AGAGAAA*G*AGCCATT-3′, residues mutated in the siRNA-resistant survivin mutants are underlined; antisense: 5′-UGGCUCUUUCUCUGUCCAGTT-3′) has been described (26). Cells were treated in parallel with a scrambled siRNA duplex

(sense: 5′-GGUGUGCUGUUUGGAGGUCTT-3′, antisense: 5′-GAACUCCAAACAGCACACCTT-3′) as a control. The siRNA duplexes (each 50 nM) were transfected together with 0.2 μg of the RFP expression plasmid using Lipofectamine2000® (Invitrogen Life Technologies) according to the manufacturer's recommendations.

Quantification of apoptosis

Apoptosis was assessed by TUNEL staining using the *in situ* cell death detection kit (Roche Diagnostics, Mannheim, Germany) as described (23). Briefly, 200 GFP-positive cells from three separate images were inspected, the number of TUNEL-positive cells was counted, and the percentages were calculated. In microinjection experiments, 100 Ab-injected cells visualized by immunofluorescence using FITC-labeled α-IgG antibodies (Sigma Aldrich) were inspected and the number of TUNEL-positive cells was determined. Cell extracts were assayed for caspase-3-dependent hydrolysis of the fluorogenic substrate N-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide (Axxora GmbH, Grünberg, Germany); enzyme-catalyzed release of *p*-nitroanilide was monitored at 405 nm as described (27).

RESULTS

Survivin contains an evolutionary conserved leucine-rich NES interacting with Crm1

The pivotal role of survivin as an apoptosis inhibitor and a regulator of cell division suggests that regulated nucleocytoplasmic transport controls the localization of survivin. We found survivin overexpressed in CRC and head and neck squamous cell carcinomas (HNSCC) compared with the corresponding non-neoplastic mucosa (NOM) of the same patient (**Fig. 1*A***, Supplemental Fig. S1*A*, and Supplemental Tables S1 and S2). Survivin was detected as a cytoplasmic but also as a predominantly nuclear protein in interphase tumor cells (Fig. 1*B*).

To analyze the nucleocytoplasmic transport of survivin in live cells, we expressed human and rat survivin (Rn_survivin-GFP) as C-terminal GFP fusion proteins (Table 1). Survivin-GFP fusions have been widely used and appear to be fully functional (12). Fluorescence microscopy shows that survivin-GFP is predominantly cytoplasmic after expression in several cell lines including the HNSCC cell line 1624 and the CRC cell line RKO (Fig. 1*C*, Supplemental Fig. S1*C*, Table 1). Treatment with the nuclear export inhibitor leptomycin B (LMB) resulted in enhanced nuclear localization (Fig. 1*C*, Supplemental Fig. S1*C*), suggesting the presence of an active NES. Although endogenous survivin was almost undetectable in most RKO and 1624 interphase cells, a similar intracellular localization and LMB sensitivity for endogenous survivin could be observed in some cells (Supplemental Fig. S1*B*).

To map potential transport signals, we expressed the survivin amino acids (aa) 1–119 and 1–88 as GFP fusions, and tested their localization and LMB sensitivity. In contrast to survivin$_{1-88}$-GFP, which was nuclear and cytoplasmic, cytoplasmic localization of survivin$_{1-119}$-GFP positioned the NES between aa 88 and 119 (Supplemental Fig. S1*D*, Table 1). We next examined the nucleocytoplasmic transport of survivin using microinjection, a highly stringent system that allows observation and quantification of transport in living cells (20). Due to the size of the GST-GFP fusion protein (54 kDa), localization of the autofluorescent transport substrate was not affected by passive diffusion upon microinjection (not depicted). In contrast, GST-survivin-GFP was quantitatively exported into the cytoplasm after nuclear injection in 1624 and RKO cells (**Fig. 2*A***, and data not shown). Export was abrogated by treatment with LMB (Fig. 2*A*). No nuclear import was observed even in the presence of LMB, arguing that survivin does not contain an active nuclear import signal (Fig. 2*A* and data not shown). Similar results were observed for rat survivin (GST-Rn_survivin-GFP) (not depicted). Subsequently, we tested the activity of potential NESs within aa 88 to 119 of survivin, matching the loosely defined leucine-rich NES-consensus sequence (22). Only a recombinant GST-GFP protein containing survivin aa 89–98 (^{89}**VKKQFEELTL**^{98}) was quantitatively exported upon microinjection (Fig. 2*B*). As a stringent control, a signal in which essential residues in the NES were mutated (NESmut; ^{89}VKKQFEELTL^{98} → ^{89}VKKQPEEATA^{98}, mutated residues underlined) was inactive under identical experimental conditions (Fig. 2*B* and

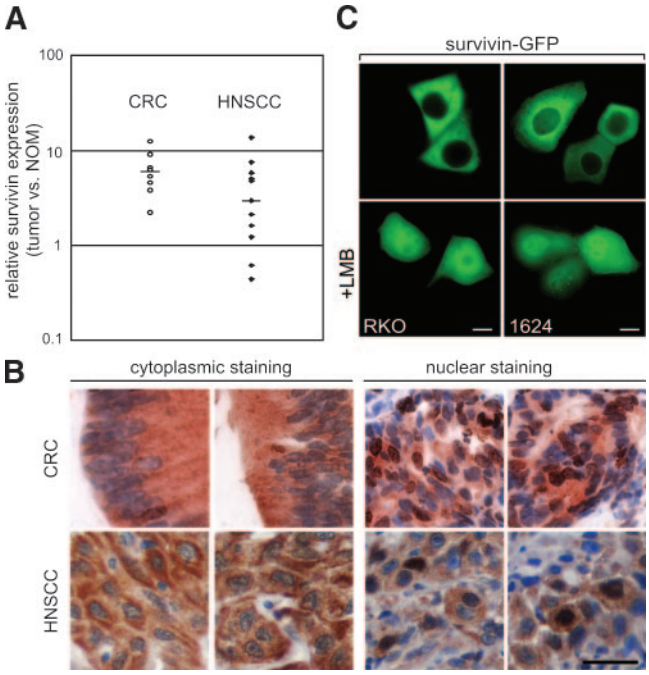

Figure 1. Survivin is overexpressed in CRC and HNSCC, and localizes to the cytoplasm and the nucleus of tumor cells. *A)* Quantitative RT-polymerase chain reaction (RT-PCR) indicating the fold change in expression of survivin in tumor *vs.* NOM. Bars, median. *B)* Representative examples of cytoplasmic and nuclear staining of survivin in tumor sections. Scale bar, 50 μm. *C)* Localization and LMB sensitivity of survivin-GFP in living transfectants. Fluorescence microscopy shows that survivin-GFP localizes predominantly to the cytoplasm, and accumulates in the nucleus on LMB treatment. Scale bars, 10 μm.

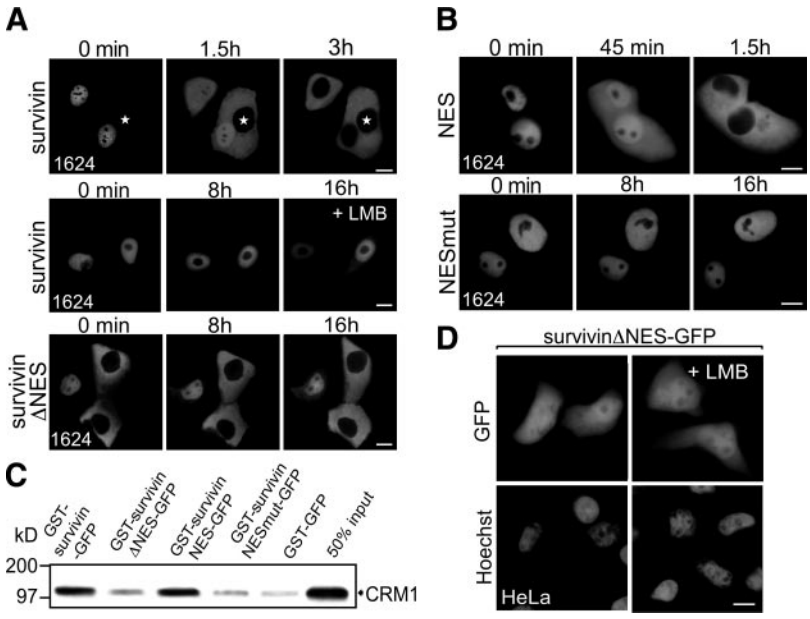

Figure 2. Survivin contains a NES interacting with Crm1. *A, B)* Indicated GST-survivin-GFP substrates were microinjected into the nucleus or cytoplasm, and transport was recorded by fluorescence microscopy. ~100 cells were injected; representative examples are shown. Scale bars, 10 μm. *A)* GST-survivin-GFP injected into nuclei was exported after 3 h (top). In the multinucleated cell, no import into the noninjected nucleus (marked by an asterisk) occurred. Export was prevented upon LMB treatment (middle). Export-deficient GST-survivin$_{\Delta NES}$-GFP was neither exported after nuclear injection nor imported after cytoplasmic injection (bottom). *B)* Nuclear-injected GST-survivinNES-GFP (aa 89–99) was efficiently exported (top) whereas inactivation of the NES (GST-survivinNESmut-GFP) blocked export (bottom). *C)* Survivin interacts with Crm1 in GST pull-down assays. *In vitro* translated [^{35}S]-labeled Crm1 protein was incubated with equal amounts of the indicated GST-GFP fusions. Binding of Crm1 to the NES-containing substrates was abolished by mutating the NES. GST-GFP served to control for unspecific binding. *D)* Ectopically expressed survivin$_{\Delta NES}$-GFP is distributed equally between the nucleus and the cytoplasm, and does not respond to LMB. Nuclei are marked by Hoechst dye. Scale bars, 10 μm.

Supplemental Fig. S1*F*). To analyze the biological relevance of the signal, we mutated the NES in the context of the full-length protein. In contrast to WT survivin, survivin$_{\Delta NES}$-GFP was distributed equally between the nucleus and the cytoplasm, and did not respond to LMB treatment (Fig. 2*D*).

Inhibition of export by treatment with LMB indicated that survivin was exported via the Crm1 pathway. The direct NES-mediated survivin/Crm1 interaction was verified biochemically by *in vitro* interaction studies. Figure 2*C* illustrates that recombinant GST-survivin-GFP binds to Crm1 in contrast to inactive GST-survivin$_{\Delta NES}$-GFP or GST-GFP alone. Binding of Crm1 was dependent on Ran-GTP (Supplemental Fig. S2*A*). Similar results were obtained for GST-survivinNES-GFP and GST-survivinNESmut-GFP. Upon overexpression, Crm1 also colocalizes with survivin-GFP but not with Survivin$_{\Delta NES}$-GFP at the nuclear membrane (Supplemental Fig. S1*G*). Similar results were obtained upon expression of untagged survivin proteins (not depicted).

These results unambiguously define the leucine-rich NES conserved in mammalian survivin proteins (Supplemental Fig. S1*E*) and exclude additional NESs.

The survivin-NES is required to promote proper cell division

Besides its role as a nuclear export receptor, Crm1 has been described as an effector for mitotic spindle assembly and functions (28). To investigate the biological significance of the survivin-Crm1 interaction, we analyzed whether survivin$_{\Delta NES}$-GFP was able to counteract the formation of multinucleated cells upon ablation of endogenous survivin by RNAi. Various reports demonstrated defects in cell cycle progression after down-regulation of survivin, resulting in mitotic arrest and polyploidy (see ref. 12). Transfection of GFP-expressing HeLa cells with survivin siRNA resulted in an increased number of multinuclear cells (**Fig. 3*B***, left panel). This effect was reduced in survivin-GFP but not in survivin$_{\Delta NES}$-GFP-expressing cell lines (middle panel), albeit both proteins were expressed equally (Fig. 3*A*). Since RNAi affected endogenous as well as ectopically expressed survivin, we generated HeLa cell lines stably expressing siRNA-resistant survivin-GFP fusion proteins (survivin_sim-GFP and survivin$_{\Delta NES}$_sim-GFP), by introducing two silent mutations within the survivin siRNA target sequence. Upon RNAi-mediated ablation of endogenous survivin, the inability of NES-deficient survivin to support proper mitosis became more obvious (Fig. 3*B*, right panel, and Supplemental Fig. S2*B*). Of note, blocking survivin's export by prolonged treatment with LMB also resulted in multinuclear cells, which finally underwent apoptosis (not depicted).

The NES is required to tether survivin and the survivin/Aurora-B complex to the mitotic machinery

To provide a molecular rationale why NES-deficient survivin was unable to promote proper cell division, we examined the localization of survivin-GFP and survivin$_{\Delta NES}$-GFP in dividing cells. In contrast to survivin-GFP, which localizes correctly to the metaphase plate, the kinetochores, and the midbody during cytokinesis, survivin$_{\Delta NES}$-GFP fails to associate with the mitotic machinery (Fig. 3*C* and data not shown; Supplemental Fig. S2*C*). Similar results were observed in the RKO cell line, as well as for untagged survivin and survivin$_{\Delta NES}$ (not depicted). The kinase Aurora-B is another essential component of the CPC, and forms a

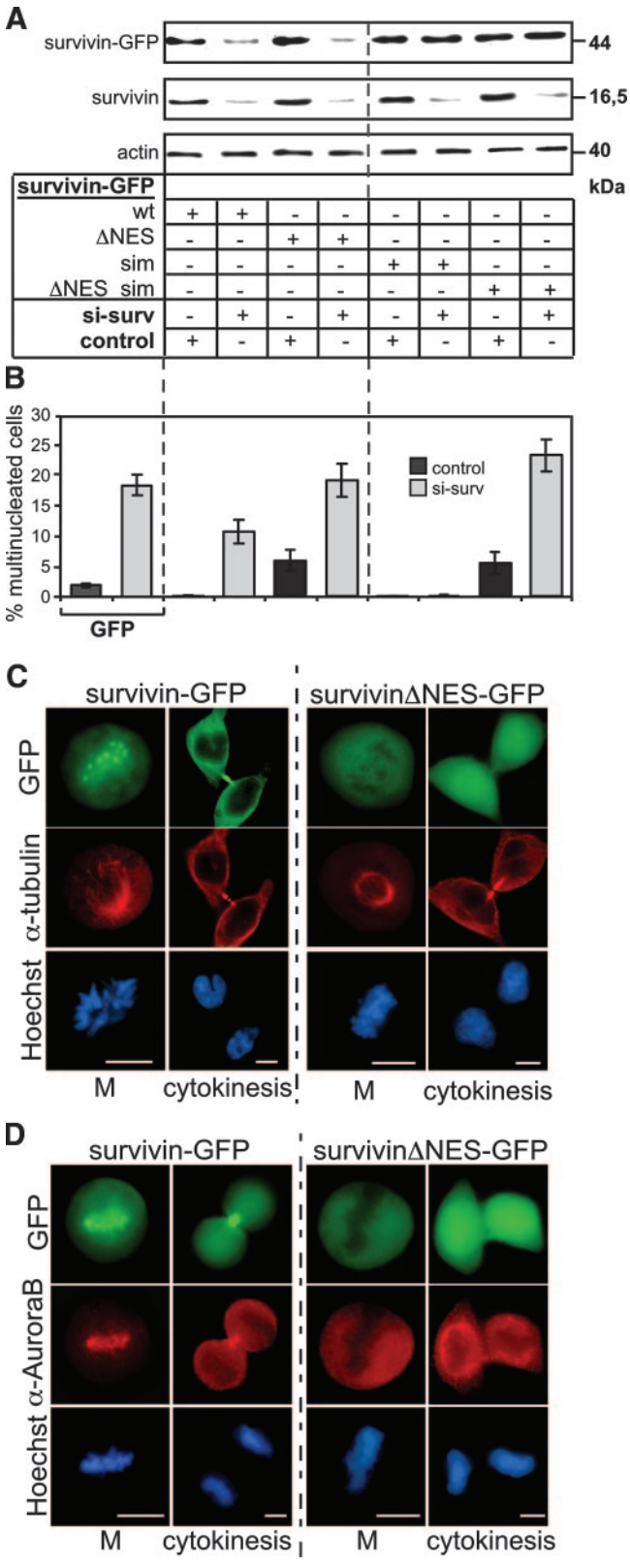

Figure 3. The integrity of the survivin NES is required to promote proper cell division. HeLa cells stably expressing GFP, survivin-GFP, survivin$_{\Delta NES}$-GFP, or the siRNA resistant survivin mutants (survivin_sim-GFP and survivin$_{\Delta NES}$_sim-GFP) were transfected with survivin siRNA or a control siRNA together with an RFP expression plasmid as the transfection control. *A)* siRNA-mediated silencing and similar expression levels of the survivin-GFP fusion proteins were verified by Western blot analysis using an α-survivin Ab. Actin served to control loading. *B)* In parallel cultures, the number of multinucleated cells was examined in 200 cells, and the percentages of GFP- and RFP-double-positive cells with two or more nuclei, was determined. Columns, mean; bars, ±SD from two independent experiments. siRNA-mediated silencing of survivin resulted in an increased number of multinuclear cells (left) that could be rescued by coexpressing survivin-GFP, but not by survivin$_{\Delta NES}$-GFP (middle). Since RNAi affected endogenous as well as ectopically expressed survivin (*A*), rescue by survivin_sim-GFP but not by survivin$_{\Delta NES}$_sim-GFP became more obvious (right). *C, D)* Export-deficient survivin fails to tether survivin and Aurora-B kinase to the mitotic machinery. *C)* Survivin-GFP correctly localizes to the metaphase plate (M) and the midbody during cytokinesis. In contrast, survivin$_{\Delta NES}$-GFP is not associated with the mitotic machinery. *D)* Aurora-B kinase correctly colocalizes with survivin-GFP at the spindle midzone and during cytokinesis. In the presence of survivin$_{\Delta NES}$-GFP, Aurora-B kinase is not detectable at either the mitotic spindle or midbody, but localizes similar to survivin$_{\Delta NES}$-GFP. DNA is marked by Hoechst dye (blue), microtubules by anti-α-tubulin staining (red), and Aurora-B kinase by α-Aurora-B kinase Ab (red). Scale bars, 10 μm.

complex with survivin. We found that Aurora-B kinase correctly colocalizes with survivin-GFP at the spindle midzone and midbody (Fig. 3*D*, left panel). In contrast, in the presence of survivin$_{\Delta NES}$-GFP, Aurora-B kinase is not detectable at the centromeres but localizes with survivin$_{\Delta NES}$-GFP (Fig. 3*D*, right panel; Supplemental Fig. S2*D*).

To determine that the observed inhibition of the biological activity of survivin is mediated by preventing the survivin-Crm1 interaction rather than by affecting survivin's capability to dimerize and to interact with Aurora-B kinase, we performed binding studies. Survivin-GFP/Aurora-B- and survivin$_{\Delta NES}$-GFP/Aurora-B-complexes could be recovered with similar efficiencies from HeLa transfectants (Supplemental Fig. S2*G*). In addition, immobilized GST-survivin-GFP or GST-survivin$_{\Delta NES}$-GFP bound *in vitro* translated survivin-GFP or Aurora-B with equal efficiencies (Supplemental Fig. S2*E, F*).

These results strongly suggest that the presence of the NES in survivin is required for guiding not only survivin but also the survivin/Aurora-B kinase complex to the mitotic machinery, underlining why the survivin NES seems to be essential for proper cell division.

The cytoprotective activity of survivin depends on active nuclear export

Survivin expression in cell lines and tumors has been correlated with resistance against chemo- and radiotherapy-induced apoptosis (4, 26). To investigate whether nuclear export was required for the cytoprotective activity of survivin, HeLa and RKO cells stably expressing survivin-GFP or survivin$_{\Delta NES}$-GFP, respectively, were treated with chemotherapeutic compounds or irradiated. We recently found that histone deacetylase inhibitors (HDACi) induced apoptosis and down-

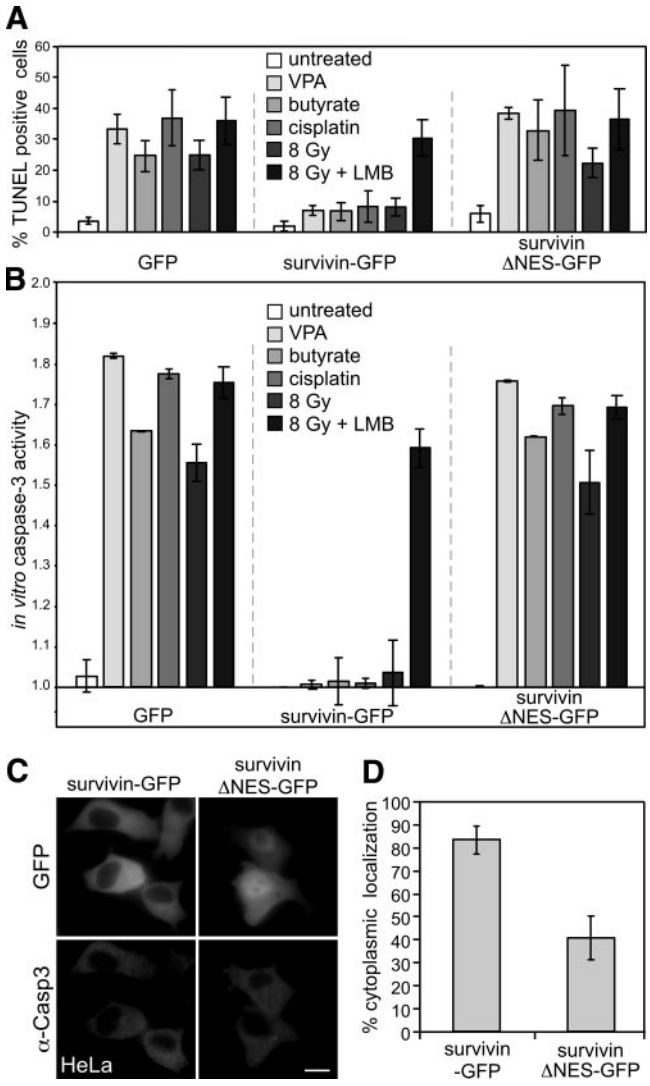

Figure 4. Nuclear export is required for survivin-mediated protection against chemo- and radiotherapy-induced apoptosis. *A, B)* HeLa cells stably expressing the indicated proteins were treated with the HDAC inhibitors VPA (1.5 mM) or sodium butyrate (1.5 mM), cisplatin (3 mM), or irradiated (8 Gy). 48 h later, apoptosis was assessed by *A*) TUNEL staining or by *B)* measuring caspase-3 activity. Caspase-3 activity in untreated survivin-GFP-expressing cells was set to 1. Columns, mean; bars, ±SD from two independent experiments. In contrast to survivin-GFP, survivin$_{\Delta NES}$-GFP could not counteract induction of apoptosis by drug treatment or irradiation. Blocking the nuclear export of survivin by treatment with LMB for 24 h enhanced irradiation-induced apoptosis. *C, D)* Nuclear export of survivin enhances potential interaction with procaspase-3. HeLa cells were transfected with the indicated plasmids and stained with an α-caspase-3 Ab. *C)* the amount of survivin protein that has the potential to interact with cytoplasmic procaspase-3 is significantly reduced for the NES mutant. Scale bar, 10 μm. *D)* Percentage of cytoplasmic survivin-GFP was quantitated in 100 procaspase-3-expressing cells. Columns, mean; bars, ±SD from two independent experiments.

regulation of survivin in tumor cells (24). **Figure 4*A, B*** shows that survivin-GFP could counteract the induction of apoptosis by treatment with the HDACi VPA and butyrate. Similar results were obtained upon treatment with the DNA-damaging agent cisplatin. In contrast, treatment of GFP or survivin$_{\Delta NES}$-GFP-expressing cells results in increased apoptosis evident by TUNEL staining and increased caspase-3 activity (Fig. 4*A*, *B*). Export-deficient survivin-GFP also fails to protect cells against irradiation-induced apoptosis. Blocking the nuclear export of survivin by treatment with LMB for 24 h enhanced irradiation-induced cell death. Similar expression levels of survivin-GFP and survivin$_{\Delta NES}$-GFP in the cell lines were controlled by immunoblot (not depicted).

IAPs are assumed to be active predominantly in the cytoplasm. For survivin, the types of caspases and the exact molecular mechanisms involved are still under investigation. We find that the amount of cytoplasmic survivin having the potential to interact with cytoplasmic procaspase-3 and -9 is significantly higher for survivin-GFP compared with survivin$_{\Delta NES}$-GFP, providing a possible explanation for the difference observed in their cytoprotective activity (Fig. 4*C*, *D* and data not shown).

Preferential nuclear localization of survivin correlates with enhanced patient survival

The above data suggest that interference with the nuclear export of survivin results in increased nuclear survivin with impaired tumor-promoting activities. Consequently, we would expect increased overall survival in patients with predominantly nuclear survivin in their tumors. To test this hypothesis, the intracellular localization of survivin was analyzed by IHC in colorectal cancer specimens (for patients' characteristics, see Supplemental Table S3). Predominantly "nuclear survivin" tumors (Fig. 1*B* depicts an example of a representative tumor) were evident in 24 of 263 cases (9.2%). Kaplan-Meier curves of overall and recurrence-free survival demonstrate a statistically significant association (P=0.005) with improved survival in these patients as calculated by the log-rank test (**Fig. 5*A***). No multivariant analysis was performed. The possibility that the detected nuclear survivin protein represents the survivin$_{\Delta exon3}$ splice variant instead of WT survivin was controlled by analyzing expression levels of the respective proteins in tumors with enhanced nuclear survivin staining by RT-qPCR. In contrast to WT survivin, survivin$_{\Delta exon3}$ levels were almost undetectable (data not shown), consistent with previous reports (7).

NES-specific antibodies inhibit nuclear export and activity of survivin

Our results indicate that blocking the nuclear export of survivin by molecular decoys could be exploited to affect survivin's functions. However, no substrate-specific export inhibitors have been identified so far. To support our concept of NES-specific export inhibition (23), we raised antibodies against the survivin NES peptide. Specificity of the antibodies was confirmed by immunofluorescence staining of survivin-GFP-expressing cells (Supplemental Fig. S3*A*). Microinjection of

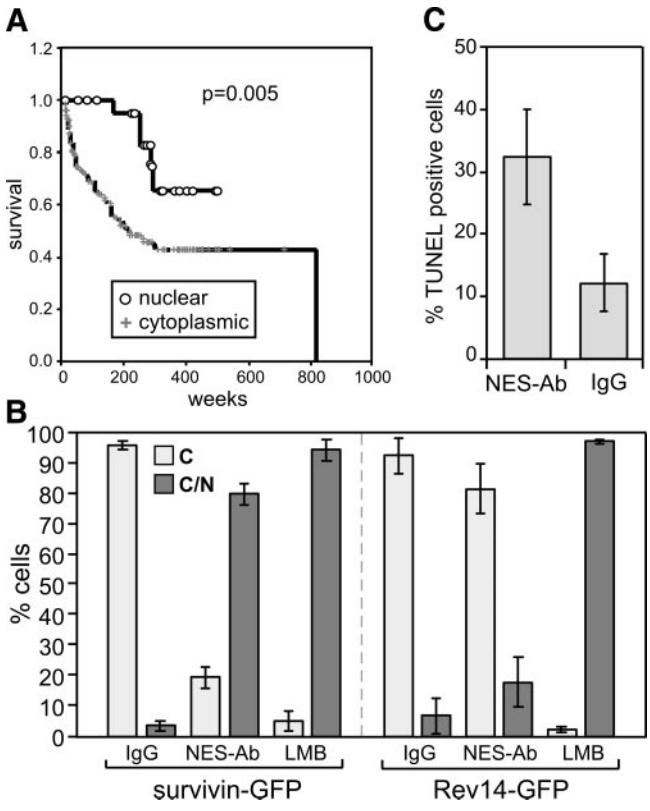

Figure 5. Nuclear survivin is associated with enhanced survival of CRC patients and can be induced by interference with nuclear export. *A)* Kaplan-Meier survival curves for positive (n=24) and negative (n=239) cases of CRC regarding "nuclear survivin" tumors. Patients with predominantly nuclear survivin staining in their tumors showed a statistically significant association (P=0.005) with improved survival as calculated by the log-rank test. *B, C)* Survivin NES-specific antibodies (NES antibody) inhibit nuclear export and the cytoprotective activity of survivin. *B)* Microinjection of the NES antibody (3 mg/ml) but not of control IgG (5 mg/ml) into the nucleus of survivin-GFP-expressing cells causes inhibition of export and nuclear accumulation. In contrast, export of the shuttle protein Rev14-GFP is not affected. As a control, LMB treatment inhibited export of both GFP fusion proteins. The intracellular localization of GFP fusion proteins was examined in 100 injected cells, and injected antibodies were visualized by staining with Cy3-labeled α-IgG. Columns, mean; bars, ±SD from two independent experiments. *C)* Injection of the NES antibody interfered with the cytoprotective activity of survivin, resulting in enhanced apoptosis. RKO cells were injected into the nucleus with the NES antibody (3 mg/ml) or control IgG (5 mg/ml) and treated with 3 mM cisplatin for 12 h postinjection. Injected cells were visualized by immunofluorescence using FITC-labeled α-IgG, and the number of apoptotic TUNEL-positive cells was determined in 100 injected cells. Columns, mean; bars, ±SD from two independent experiments.

the NES antibody (NES-ab) into the nucleus of survivin-GFP-expressing cells results in inhibition of export and subsequent nuclear accumulation of survivin-GFP (Fig. 5*B* and Supplemental Fig. S3*B*). In contrast, shuttling of a NES-containing HIV-1 Rev mutant (Rev14-GFP) (29) is not affected (Fig. 5*B* and Supplemental Fig. S3*B*), underlining the NES specificity of the survivin NES antibody. Subsequently, the NES antibody was injected into the nuclei of RKO cells and cells were treated with cisplatin. TUNEL staining revealed that NES antibody-injected cells showed a significantly higher apoptotic rate than control cells injected with IgG (Fig. 5*C*).

DISCUSSION

Survivin is overexpressed in most tumors and appears to be involved in tumorigenesis and clinical outcome, which justifies its role as a rational target for cancer therapy. We show here that survivin's dynamic intracellular localization is regulated by active nuclear export, which appears to be essential for survivin's tumor-promoting functions.

Whereas Rodriguez *et al.* (30) proposed that survivin's export is mediated in *trans*-by a NES-containing protein binding to the carboxyl-terminal domain of survivin, we identified the NES. The NES of survivin is conserved in mammalian survivin proteins and fits the consensus sequence for leucine-rich export signals (22). Export of survivin is mediated by Crm1, since Crm1 antagonists cause nuclear accumulation of survivin, block export of recombinant survivin, and survivin-GFP interacts with Crm1 *in vitro* and *in vivo*. Although suggested previously (31), our data do not support the presence of an active nuclear import signal in survivin. For one NES mutation or LMB treatment do not result in complete nuclear accumulation of survivin, and GST-survivin-GFP is not imported into the nucleus. Therefore, the low molecular weight of survivin even as a dimer appears to allow survivin to enter the nucleus by passive diffusion. The reported nuclear accumulation of survivin on irradiation and its potential function in postirradiation DNA damage repair (26, 32) may thus be explained by interaction with nuclear binding sites. Hence, our results indicate that interference with active nuclear import will most likely not be applicable to impede potential nuclear functions of survivin.

In contrast, it seems conceivable that inhibiting the nuclear export of survivin is a more promising strategy to affect survivin's activity. First, during cell division, the NES appears to be required to tether not only survivin but also the survivin/Aurora-B kinase complex to the mitotic machinery. One can speculate that among components of the CPC, Crm1 may be critically involved in guiding survivin during mitosis. Besides its function as a nuclear export receptor, Crm1 has recently been identified as an essential mitotic effector (28), and further studies are required to dissect the molecular mechanism. Second, we observed that survivin (but not export-deficient survivin) is able to efficiently counteract chemo- and radiotherapy induced apoptosis. We consider these findings to be potentially relevant clinically since HDAC inhibitors, cisplatin compounds, and irradiation are current treatment modalities for HNSCC and CRC patients, and therapy resistance represents a general problem in

oncology. Since the exact mechanism by which survivin directly or indirectly affects the apoptotic machinery in the cytoplasm has not been resolved (33), enhancing the cytoplasmic concentration of survivin by active nuclear export may promote survivin's antiapoptotic function. Thus, preferential nuclear localization of survivin in tumors should be linked to a favorable prognosis as shown for colorectal cancer (this study), breast cancer (34), non-small-cell lung cancer (35), ovary tumors (36), and pancreatic cancer (37). Although, some reports consider nuclear survivin to be associated with poor survival (38, 39), this discrepancy may be due to the variable parameters used to classify a tumor as "nuclear survivin" *vs.* "cytoplasmic survivin." The molecular reasons why survivin accumulates in some tumors in contrast to others are not yet known. Our previous studies (22) indicate that nuclear localization of shuttle proteins can be induced by the competition for export factors or by interfering with the nuclear transport machinery (17). In addition, mutations in the NES of survivin or binding to nuclear components could account for the pronounced nuclear localization observed in patient material.

Although we find that export inhibition by LMB increased irradiation-induced apoptosis, and LMB has also been proposed for anticancer therapy (40), Crm1-directed inhibitors can not be used in therapeutic applications due to their toxic side effects by blocking all Crm1-mediated transport pathways. Therefore, protein-specific transport inhibitors are urgently needed. Since NESs can be grouped into specific categories according to their activity *in vivo* (22, 23), these differences represent an attractive opportunity to selectively block export and the biological functions of proteins by the generation of NES-specific inhibitors. In our study, the application of the survivin-NES directed antibodies interfered with survivin's export and function, and supports our concept of NES-specific interference. These results and the availability of our recently developed cell based translocation assays (23) encourage screens to identify NES-specific small molecules. Positioning the linear NES in the NMR structure of the survivin dimer (6) (Supplemental Fig. S3*C*) may help to design NES binding inhibitors *in silico*. Since survivin is not only expressed in somatic cancer cells but also in tumor endothelial (41) and cancer stem cells (42), survivin-specific transport inhibitors may have a promising therapeutic potential. FJ

We thank Gert Carra and Sandra Schneider for excellent technical assistance, and Bernd Groner for support. Grant support: BMBF NGFN initiative (N1KR-S31T24), EU-FP6 (BRECOSM), the Studienstiftung des deutschen Volkes (S.K.) and the Stiftung Tumorforschung Kopf-Hals.

REFERENCES

1. Hanahan, D., and Weinberg, R. A. (2000) The hallmarks of cancer. *Cell* **100,** 57–70
2. Vogelstein, B., and Kinzler, K. W. (2004) Cancer genes and the pathways they control. *Nat. Med.* **10,** 789–799
3. Okada, H., and Mak, T. W. (2004) Pathways of apoptotic and non-apoptotic death in tumour cells. *Nat. Rev. Cancer* **4,** 592–603
4. Altieri, D. C. (2003) Validating survivin as a cancer therapeutic target. *Nat. Rev. Cancer* **3,** 46–54
5. Salvesen, G. S., and Duckett, C. S. (2002) IAP proteins: blocking the road to death's door. *Nat. Rev. Mol. Cell. Biol.* **3,** 401–410
6. Sun, C., Nettesheim, D., Liu, Z., and Olejniczak, E. T. (2005) Solution structure of human survivin and its binding interface with smac/diablo. *Biochemistry* **44,** 11–17
7. Li, F. (2005) Role of survivin and its splice variants in tumorigenesis. *Br. J. Cancer* **92,** 212–216
8. Dohi, T., Okada, K., Xia, F., Wilford, C. E., Samuel, T., Welsh, K., Marusawa, H., Zou, H., Armstrong, R., Matsuzawa, S., *et al.* (2004) An IAP-IAP complex inhibits apoptosis. *J. Biol. Chem.* **279,** 34087–34090
9. Shin, S., Sung, B. J., Cho, Y. S., Kim, H. J., Ha, N. C., Hwang, J. I., Chung, C. W., Jung, Y. K., and Oh, B. H. (2001) An anti-apoptotic protein human survivin is a direct inhibitor of caspase-3 and -7. *Biochemistry* **40,** 1117–1123
10. O'Connor, D. S., Grossman, D., Plescia, J., Li, F., Zhang, H., Villa, A., Tognin, S., Marchisio, P. C., and Altieri, D. C. (2000) Regulation of apoptosis at cell division by p34cdc2 phosphorylation of survivin. *Proc. Natl. Acad. Sci. U. S. A.* **97,** 13103–13107
11. Fortugno, P., Beltrami, E., Plescia, J., Fontana, J., Pradhan, D., Marchisio, P. C., Sessa, W. C., and Altieri, D. C. (2003) Regulation of survivin function by Hsp90. *Proc. Natl. Acad. Sci. U. S. A.* **100,** 13791–13796
12. Vagnarelli, P., and Earnshaw, W. C. (2004) Chromosomal passengers: the four-dimensional regulation of mitotic events. *Chromosoma* **113,** 211–222
13. Maiato, H., DeLuca, J., Salmon, E. D., and Earnshaw, W. C. (2004) The dynamic kinetochore-microtubule interface. *J. Cell Sci.* **117,** 5461–5477
14. Ferrando-May, E. (2005) Nucleocytoplasmic transport in apoptosis. *Cell Death Differ.* **12,** 1263–1276
15. Weis, K. (2003) Regulating access to the genome: nucleocytoplasmic transport throughout the cell cycle. *Cell* **112,** 441–451
16. Xu, L., and Massague, J. (2004) Nucleocytoplasmic shuttling of signal transducers. *Nat. Rev. Mol. Cell. Biol.* **5,** 209–219
17. Kau, T. R., Way, J. C., and Silver, P. A. (2004) Nuclear transport and cancer: from mechanism to intervention. *Nat. Rev. Cancer* **4,** 106–117
18. Schlingemann, J., Habtemichael, N., Ittrich, C., Toedt, G., Kramer, H., Hambek, M., Knecht, R., Lichter, P., Stauber, R. H., and Hahn, M. (2005) Patient-based cross-platform comparison of oligonucleotide microarray expression profiles. *Lab. Invest.* **85,** 1024–1039
19. Knosel, T., Yu, Y., Stein, U., Schwabe, H., Schluns, K., Schlag, P. M., Dietel, M., and Petersen, I. (2004) Overexpression of cyclooxygenase-2 correlates with chromosomal gain at the cyclooxygenase-2 locus and decreased patient survival in advanced colorectal carcinomas. *Dis. Colon Rectum* **47,** 70–77
20. Knauer, S. K., Carra, G., and Stauber, R. H. (2005) Nuclear export is evolutionarily conserved in CVC paired-like homeobox proteins and influences protein stability, transcriptional activation, and extracellular secretion. *Mol. Cell. Biol.* **25,** 2573–2582
21. Wheatley, S. P., Henzing, A. J., Dodson, H., Khaled, W., and Earnshaw, W. C. (2004) Aurora-B phosphorylation in vitro identifies a residue of survivin that is essential for its localization and binding to inner centromere protein (INCENP) in vivo. *J. Biol. Chem.* **279,** 5655–5660
22. Heger, P., Lohmaier, J., Schneider, G., Schweimer, K., and Stauber, R. H. (2001) Qualitative highly divergent nuclear export signals can regulate export by the competition for transport cofactors in vivo. *Traffic* **2,** 544–555
23. Knauer, S. K., Moodt, S., Berg, T., Liebel, U., Pepperkok, R., and Stauber, R. H. (2005) Translocation biosensors to study signal specific nucleo-cytoplasmic transport, protease activity and protein interactions. *Traffic* **6,** 1–13
24. Bug, G., Ritter, M., Wassmann, B., Schoch, C., Heinzel, T., Schwarz, K., Romanski, A., Kramer, O. H., Kampfmann, M., Hoelzer, D., *et al.* (2005) Clinical trial of valproic acid and all-trans retinoic acid in patients with poor-risk acute myeloid leukemia. *Cancer* **104,** 2717–2725

25. Dalken, B., Giesubel, U., Knauer, S. K., and Wels, W. S. (2006) Targeted induction of apoptosis by chimeric granzyme B fusion proteins carrying antibody and growth factor domains for cell recognition. *Cell Death Differ.* **13,** 576–585
26. Rodel, F., Hoffmann, J., Distel, L., Herrmann, M., Noisternig, T., Papadopoulos, T., Sauer, R., and Rodel, C. (2005) Survivin as a radioresistance factor, and prognostic and therapeutic target for radiotherapy in rectal cancer. *Cancer Res.* **65,** 4881–4887
27. Kramer, O. H., Baus, D., Knauer, S. K., Stein, S., Jager, E., Stauber, R. H., Grez, M., Pfitzner, E., and Heinzel, T. (2006) Acetylation of Stat1 modulates NF-{kappa}B activity. *Genes Dev.* **20,** 473–485
28. Arnaoutov, A., Azuma, Y., Ribbeck, K., Joseph, J., Boyarchuk, Y., Karpova, T., McNally, J., and Dasso, M. (2005) Crm1 is a mitotic effector of Ran-GTP in somatic cells. *Nat. Cell Biol.* **7,** 626–632
29. Stauber, R. H., Afonina, E., Gulnik, S., Erickson, J., and Pavlakis, G. N. (1998) Analysis of intracellular trafficking and interactions of cytoplasmic HIV-1 Rev mutants in living cells. *Virology* **251,** 38–48
30. Rodriguez, J. A., Span, S. W., Ferreira, C. G., Kruyt, F. A., and Giaccone, G. (2002) CRM1-mediated nuclear export determines the cytoplasmic localization of the antiapoptotic protein Survivin. *Exp. Cell Res.* **275,** 44–53
31. Li, F., Yang, J., Ramnath, N., Javle, M. M., and Tan, D. (2005) Nuclear or cytoplasmic expression of survivin: what is the significance? *Int. J. Cancer* **114,** 509–512
32. Chakravarti, A., Zhai, G. G., Zhang, M., Malhotra, R., Latham, D. E., Delaney, M. A., Robe, P., Nestler, U., Song, Q., and Loeffler, J. (2004) Survivin enhances radiation resistance in primary human glioblastoma cells via caspase-independent mechanisms. *Oncogene* **23,** 7494–7506
33. Dohi, T., and Altieri, D. C. (2005) Mitochondrial dynamics of survivin and "four dimensional" control of tumor cell apoptosis. *Cell Cycle* **4**
34. Kennedy, S. M., O'Driscoll, L., Purcell, R., Fitz-Simons, N., McDermott, E. W., Hill, A. D., O'Higgins, N. J., Parkinson, M., Linehan, R., and Clynes, M. (2003) Prognostic importance of survivin in breast cancer. *Br. J. Cancer* **88,** 1077–1083
35. Vischioni, B., van der Valk, P., Span, S. W., Kruyt, F. A., Rodriguez, J. A., and Giaccone, G. (2004) Nuclear localization of survivin is a positive prognostic factor for survival in advanced non-small-cell lung cancer. *Ann. Oncol.* **15,** 1654–1660
36. Ferrandina, G., Legge, F., Martinelli, E., Ranelletti, F. O., Zannoni, G. F., Lauriola, L., Gessi, M., Gallotta, V., and Scambia, G. (2005) Survivin expression in ovarian cancer and its correlation with clinico-pathological, surgical and apoptosis-related parameters. *Br. J. Cancer* **92,** 271–277
37. Tonini, G., Vincenzi, B., Santini, D., Scarpa, S., Vasaturo, T., Malacrino, C., Coppola, R., Magistrelli, P., Borzomati, D., Baldi, A., *et al.* (2005) Nuclear and cytoplasmic expression of survivin in 67 surgically resected pancreatic cancer patients. *Br. J. Cancer* **92,** 2225–2232
38. Shinohara, E. T., Gonzalez, A., Massion, P. P., Chen, H., Li, M., Freyer, A. S., Olson, S. J., Andersen, J. J., Shyr, Y., Carbone, D. P., *et al.* (2005) Nuclear survivin predicts recurrence and poor survival in patients with resected nonsmall cell lung carcinoma. *Cancer* **103,** 1685–1692
39. Grabowski, P., Griss, S., Arnold, C. N., Horsch, D., Goke, R., Arnold, R., Heine, B., Stein, H., Zeitz, M., and Scherubl, H. (2005) Nuclear survivin is a powerful novel prognostic marker in gastroenteropancreatic neuroendocrine tumor disease. *Neuroendocrinology* **81,** 1–9
40. Vigneri, P., and Wang, J. Y. (2001) Induction of apoptosis in chronic myelogenous leukemia cells through nuclear entrapment of BCR-ABL tyrosine kinase. *Nat. Med.* **7,** 228–234
41. Blanc-Brude, O. P., Mesri, M., Wall, N. R., Plescia, J., Dohi, T., and Altieri, D. C. (2003) Therapeutic targeting of the survivin pathway in cancer: initiation of mitochondrial apoptosis and suppression of tumor-associated angiogenesis. *Clin. Cancer Res.* **9,** 2683–2692
42. Kim, P. J., Plescia, J., Clevers, H., Fearon, E. R., and Altieri, D. C. (2003) Survivin and molecular pathogenesis of colorectal cancer. *Lancet* **362,** 205–209

Received for publication February 12, 2006.
Accepted for publication August 14, 2006.