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## Chk2 Phosphorylation of Survivin-ΔEx3 Contributes to a DNA Damage–Sensing Checkpoint in Cancer

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### Abstract

Survivin is an oncogene that functions in cancer cell cytoprotection and mitosis. Here we report that differential expression in cancer cells of a C-terminal splice variant of survivin, termed survivin-ΔEx3, is tightly associated with aggressive disease and markers of unfavorable prognosis. In contrast to other survivin variants, survivin-ΔEx3 localized exclusively to nuclei in tumor cells and was phosphorylated at multiple residues by the checkpoint kinase Chk2 during DNA damage. Mutagenesis of the Chk2 phosphorylation sites enhanced the stability of survivin-ΔEx3 in tumor cells, inhibited the expression of phosphorylated H2AX ( $\gamma$ H2AX) in response to double-strand DNA breaks, and impaired growth after DNA damage. DNA damage induced Chk2 phosphorylation, stabilization of p53, induction of the cyclin-dependent kinase inhibitor p21, and homologous recombination-induced repair were not affected. *In vivo*, active Chk2 was detected at the earliest stages of the colorectal adenoma-to-carcinoma transition, persisted in advanced tumors, and correlated with increased survivin expression. Together, our findings suggest that Chk2-mediated phosphorylation of survivin-ΔEx3 contributes to a DNA damage–sensing checkpoint that may affect cancer cell sensitivity to genotoxic therapies. *Cancer Res*; 72(13); 3251–9. ©2012 AACR.

### Introduction

As a unique member of the inhibitor of apoptosis gene family, survivin has attracted attention for its multiple roles in cell proliferation and cell death (1), and its abundant distribution in every human tumor, compared with normal tissues (2). Disparate oncogenic pathways control the differential expression of survivin in cancer (3), and influence its localization to multiple subcellular compartments (4, 5). Accordingly, transcription of the *survivin* locus is complex, giving rise to at least 5 mRNA species that encode, in addition to wild-type (WT) survivin, the variants survivin-2B, -3B, -2α,

and -ΔEx3 (6, 7). Structurally, survivin-2α and -3B are generated by "read-through" into intron 2 (8), or via inclusion of an alternative exon 3B (9), whereas survivin-2B and -ΔEx3 originate from the insertion of an alternative exon 2B (10), or the skipping of exon 3 (11), respectively.

Elucidating the function(s) of the survivin-spliced variants has been challenging, given their low level of expression in most cells, and the limited availability of isoform-specific reagents. For instance, survivin-2B has been reported to promote apoptosis, *in vitro* (10, 12). However, low levels of survivin-2B correlate with better survival in acute myeloid leukemia (13), and its silencing in ovarian cancer has been linked to greater sensitivity to taxanes (14). A role of the survivin isoforms in mitosis has been equally controversial, as this function has been proposed in some reports (9, 10), but negated in others (15).

In this study, we took a multidisciplinary approach of genome-wide bioinformatics, analysis of the DNA damage response, and evaluation of primary patient samples to dissect a potential role of survivin-ΔEx3 in cancer (6, 7). We found that survivin-ΔEx3 is a nuclear substrate of the checkpoint kinase, Chk2 (16) in its unique –COOH terminus (6, 7), and that this pathway contributes to a DNA damage–sensing checkpoint in tumor cells (17).

### Materials and Methods

#### Bioinformatics analysis

Fourteen cancer-related datasets with a total of 702 samples assayed on GPL5188 (Affymetrix Human Exon 1.0 ST) arrays were examined for expression of survivin-ΔEx3 (6, 7). Of the 14 datasets, 9 compared cancer or cancer-related cells with

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**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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normal controls, and 5 compared either different cancers or the same cancer at different stages (Supplementary Table S1).

The HuEx-1\_0-st Affymetrix microarray platform contains 22 probesets designed to detect sequences derived from 3 isoforms of the *survivin* locus (Fig. 1A): NM\_001168 (survivin), NM\_001012270 (survivin- $\Delta$ Ex3), and NM\_001012271 (survivin-2B). Of the 22 probesets, 9 were retained in all 14 datasets and 13 were removed because of expression below background. Probesets 3 and 16 were also removed as their expression profiles were the same as 6 other probesets that targeted the same isoforms. Of the remaining probesets (Fig. 1A), 8 of 9 probes targeted regions that were common to all 3 survivin isoforms. Probeset 9 specifically targets exon 3, which is deleted in survivin- $\Delta$ Ex3 (6, 7). Specific expression of survivin- $\Delta$ Ex3 was calculated as the difference between the average expression of the 8 common survivin probesets and probeset 9.

### Cell culture and antibodies

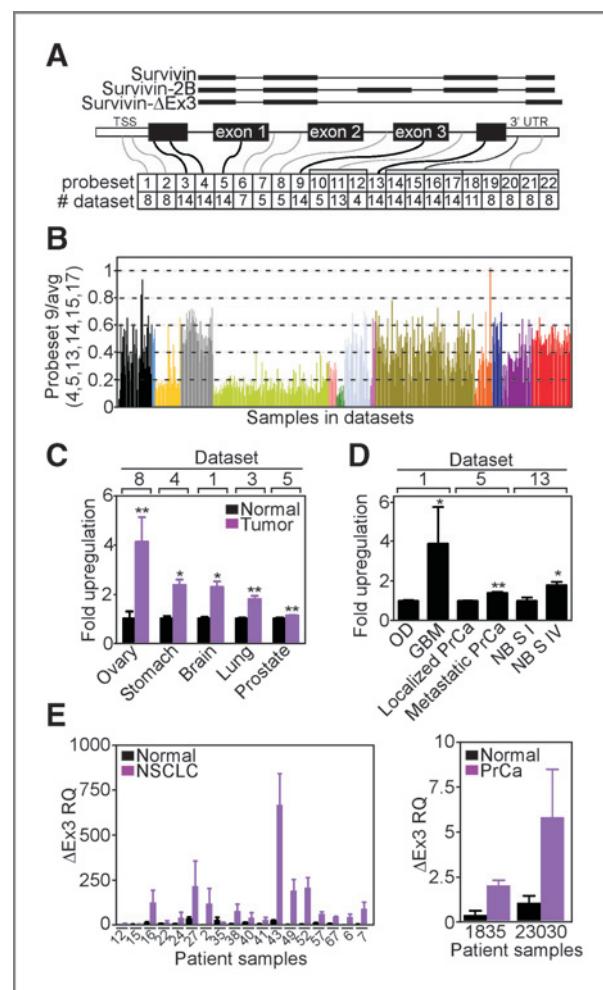
Human lung adenocarcinoma H460, breast adenocarcinoma MDA-431 and MCF-7, glioblastoma LN229, and colorectal adenocarcinoma HCT116 and SW480 cells were obtained from the American Type Culture Collection. HCT116-DR-GFP cells were kindly provided by Dr. S. Powell (Memorial Sloan Kettering Cancer Center, New York, NY). Consistent with editorial guidelines, all cell lines were used within 6 months of receipt from the cell bank. The following antibodies to Chk2 (Santa Cruz), Thr68 phosphorylated Chk2 (Cell Signaling), survivin (Novus Biologicals), p53 (Calbiochem), Ser15-phosphorylated p53 (Cell Signaling), p21 (Calbiochem), Ser139-phosphorylated histone H2AX, that is  $\gamma$ H2AX (Millipore), Aurora B (Bethyl Laboratories), Alexa Fluor 488 (Invitrogen), FLAG (Sigma-Aldrich),  $\beta$ -actin (Sigma-Aldrich), COX-IV (Cell Signaling), and RCC1 (Santa Cruz) were used.

### Mutagenesis

Substitution of predicted Chk2 phosphorylation sites Thr79 $\rightarrow$ Ala, Thr127 $\rightarrow$ Ala, and Ser98 $\rightarrow$ Ala in the unique -COOH terminus of survivin- $\Delta$ Ex3 was carried out using QuikChange Site-Directed Mutagenesis Kit (Stratagene) with oligonucleotides (mutated sequences underlined): 5'-ATGCAAAGAACCAGGAACCAGCAATAAGAAGAAAGAAT-3' (Thr79, ACA $\rightarrow$ GCA), 5'-TTATTCCCTGGTGCCGGCCAGCAGCTTCCTGT-GGGC-3' (Thr127, ACC $\rightarrow$ GCC), and 5'-AATCCATGGCAGCCA-GGCGCTCGATGGCACGGC-3' (Ser98, AGC $\rightarrow$ GCC). Mutant constructs were confirmed by DNA sequencing.

### Transfections

Tumor cell types ( $10^5$ /well) were transfected with FLAG-tagged cDNAs in the presence of lipofectamine 2000 (Invitrogen) and 250  $\mu$ L Opti-MEM I (Invitrogen) per well (18). In some experiments, HCT116 transfectants were treated with or without etoposide (2.5  $\mu$ mol/L), immunoprecipitated with an antibody to FLAG (2  $\mu$ g) plus protein G-Sepharose, and immune complexes were analyzed with an antibody to phosphorylated Ser residues (Upstate-Millipore) by Western blotting. Gene silencing by small interfering RNA (siRNA) was carried out with nontargeting or SMART pool siRNA to Chk2 (Dharmacon). Two survivin- $\Delta$ Ex3-specific siRNA sequences,



**Figure 1.** Genome-wide bioinformatics analysis of survivin- $\Delta$ Ex3 in cancer. **A**, schematic diagram of the genomic organization of WT survivin, survivin-2B, and - $\Delta$ Ex3 (top), and distribution of probesets and datasets (bottom). **B**, ratio between probeset 9 targeting WT survivin and survivin-2B, and average expression of probesets targeting all survivin isoforms (expected ratio < 1). The different colors indicate samples from 14 different datasets, incremental from left to right. **C**, fold upregulation of survivin- $\Delta$ Ex3 in tumors relative to matched normal tissues. The number of cases in normal (N) or tumor (T) groups is: ovary (N,  $n = 31$ ; T,  $n = 8$ ); stomach (N,  $n = 25$ ; T,  $n = 25$ ); brain (N,  $n = 6$ ; T,  $n = 49$ ); lung (N,  $n = 20$ ; T,  $n = 20$ ); prostate (N,  $n = 29$ ; T,  $n = 150$ ). Mean  $\pm$  SEM. The statistical analysis for differential expression of WT survivin in tumors versus normal samples is: ovary,  $P = 3 \times 10^{-7}$ ; stomach,  $P = 3 \times 10^{-5}$ ; brain,  $P = 3 \times 10^{-7}$ ; lung,  $P = 0.02$ ; prostate,  $P = 1 \times 10^{-5}$ . **D**, increased expression of survivin- $\Delta$ Ex3 during tumor progression. GBM, grade IV glioblastoma; NB, neuroblastoma; OD, oligodendrogloma; PrCa, prostate cancer. The number of cases per each condition is: OD,  $n = 2$ ; GBM,  $n = 28$ ; localized PrCa,  $n = 131$ ; metastatic PrCa,  $n = 19$ ; stage (S) I NB,  $n = 10$ ; stage (S) IV NB,  $n = 37$ ; mean  $\pm$  SEM. The statistical analysis for differential expression of WT survivin in tumors of advanced- versus early-stage is: GBM versus OD,  $P = 0.09$ ; metastatic versus localized prostate cancer,  $P = 2 \times 10^{-11}$ ; NB S4 versus NB S1,  $P = 0.002$ . **E**, primary samples of non-small cell lung cancer (NSCLC, left) or prostate cancer (PrCa, right), and matched normal tissues (NSCLC,  $n = 18$ ; PrCa,  $n = 2$ ) were analyzed for differential expression of survivin- $\Delta$ Ex3 mRNA, by quantitative RT-PCR; mean  $\pm$  SEM.

5'-GAAGAAAGAAUUUGAGGAAUU-3' and 5'-GCAAAGGAAA-CCAACAUUUU-3', were used.

### Protein expression and purification

Recombinant GST fusion proteins were expressed in *Escherichia coli* as described (19).

### Protein analysis and turnover

Tumor cell types ( $6-7 \times 10^7$ ) were fractionated in mitochondrial and cytosolic extracts as described (20). In some experiments, extracts (5 µg) from HCT116 transfectants were separated on 13% acrylamide:bis-acrylamide, 29:1 gels in the presence of 20 µmol/L Phos-tag acrylamide AAL-107M (Wako) containing 3% MeOH and 40 µmol/L MnCl<sub>2</sub>. Pull down experiments with recombinant proteins (10 µg) were carried out as described (19). For protein stability, HCT116 transfectants were treated with 0.1 mg/mL cycloheximide, harvested after 0, 2, 4, and 6 hours, and analyzed by Western blotting.

### Kinase assays

Recombinant proteins were incubated with human active Chk2 (R&D Systems, 0.1 µg/µL) in kinase buffer (pH 7.2) containing 25 mmol/L MOPS, 12.5 mmol/L β-glycerophosphate, 25 mmol/L MgCl<sub>2</sub>, 5 mmol/L EGTA, 2 mmol/L EDTA, 0.25 mmol/L DTT, and 0.1 mmol/L ATP in the presence or absence of <sup>32</sup>P-γATP (Amersham) for 20 minutes at 30°C, and detection of phosphorylated bands by autoradiography. Recombinant Cdc25 was a control Chk2 substrate.

### γH2AX reactivity

For analysis of DNA damage, tumor cell types were treated with etoposide (2.5 µmol/L) or camptothecin (CPT, 2.5 µmol/L) for 16 hours at 37°C, followed by 4 or 8 hours recovery. Cells ( $1 \times 10^6$ ) were fixed in 1% paraformaldehyde for 15 minutes and 70% ethanol for 30 minutes, permeabilized in 0.2% Triton X-100 plus 1% BSA for 10 minutes, and incubated with an antibody to γH2AX for 90 minutes. After addition of Alexa Fluor 488, cells were stained with propidium iodide (PI), and analyzed by multiparametric flow cytometry. Alternatively, cells grown on glass coverslips were processed as above and analyzed by fluorescence microscopy for γH2AX-containing foci formation using a Nikon E600 upright microscope. Fluorescence intensity and number of foci were quantified using Image J software.

### Homologous double-strand DNA break repair assay

A pDR-GFP plasmid that uses 2 modified GFP genes (I-SceI-GFP and iGFP) to create a recombination reporter was used (21). Stable HCT116-pDR-GFP clones were cotransfected with pCMV-I-SceI alone or with various cDNAs, and analyzed after 3 days by multiparametric flow cytometry (22).

### Soft agar colony formation

HCT116 transfectants ( $5 \times 10^5$ ) treated with or without etoposide (1.25 µmol/L) were analyzed for colony formation in semisolid medium, as described (20). Colony size ( $\leq 100$  or 100–200 µm) was quantified using NIH Image J software.

### Patient samples and reverse transcriptase-PCR

All patient-related investigations were approved by an Institutional Review Board. Histologically confirmed tumor and matched normal tissue samples from chemo-naive patients with diagnosis of non-small cell lung cancer ( $n = 18$ ), or prostate cancer ( $n = 2$ ) were analyzed for differential expression of survivin-ΔEx3 mRNA by RT-PCR using primers 5'-TGGAGGCTGGGAGCCA (*forward*) and 5'-AGAAAGAATTGAGGAAACTCGCGGA-3' (*reverse*). Relative levels of survivin-ΔEx3 mRNA were quantified using β2-microglobulin (β-2M, Assay-on-Demand chemistry, Hs 9999907\_m1) as a reference gene and the 2<sup>-DDCt</sup> formula.

### Histology

Anonymous primary human tissue samples representative of different stages of the colorectal adenoma-to-carcinoma transition (hyperplasia, moderate or severe dysplasia, or adenocarcinoma) were analyzed for expression of survivin, Chk2, Thr68-phosphorylated Chk2, or p53, by immunohistochemistry (20). Twenty cases per condition were scored, and a cutoff of 5% positively stained cells was used.

### Statistical analysis

Data were analyzed using the unpaired *t* test on a GraphPad software package for Windows (Prism 4.0). Differences in survivin-ΔEx3 gene expression in datasets with paired cancer/control samples (datasets 3 and 4) were analyzed using the 2-tailed *t* test. Additional comparisons were done using the Spearman correlation test. The level of significance was set at  $P < 0.05$ .

## Results

### Differential expression of survivin-ΔEx3 in human cancer

We used a genome-wide bioinformatics approach to examine the expression of survivin-ΔEx3 in cancer-associated GEO datasets (Supplementary Table S1). A schematic diagram of the genomic organization of the *survivin* gene, and its relationship to the probesets on the arrays are shown in Fig. 1A. By principal component analysis (Supplementary Fig. S1A), the expression of probeset 9 (deleted in survivin-ΔEx3) differed from that of other 8 probesets common to other survivin isoforms, validating the ability of the microarray platform to distinguish between survivin isoforms that either contain or lack exon 3 (Supplementary Fig. S1A). A hierarchical clustering based on differential probeset expression is shown in Supplementary Fig. S1B. Given the average expression of probesets 4, 5, 13, 14, 15, and 17 ( $X$  = cumulative expression of all 3 isoforms) and probeset 9 ( $Y$  = expression of survivin and survivin-2B, but not survivin-ΔEx3), we determine that  $Y < X$  for the majority of samples (Fig. 1B). Calculated as the difference  $X - Y$  and then log<sub>2</sub>-transformed, survivin-ΔEx3 mRNA was expressed at significantly higher levels in tumors of the ovary, stomach, brain, lung, and prostate compared with matched normal tissues (Fig. 1C). WT survivin was also differentially expressed in tumor versus normal tissues in the same datasets (Fig. 1; ref. 2).

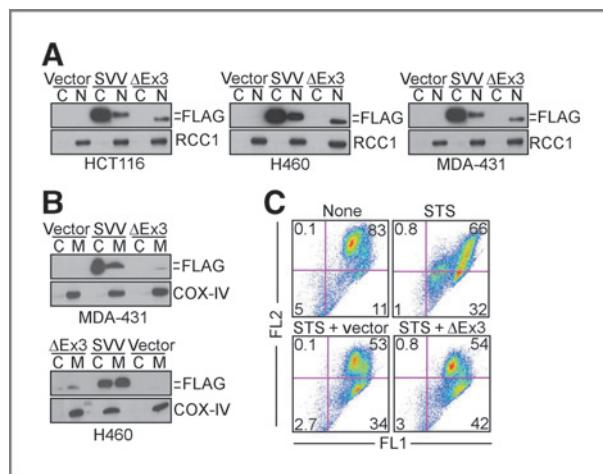
Survivin-ΔEx3 was expressed at higher levels in grade IV glioblastoma compared with low-grade oligodendrogiomas,

metastatic compared with localized prostate cancer, and stage 4 compared with stage 1 neuroblastoma (Fig. 1D; Supplementary Table S2). Survivin- $\Delta$ Ex3 expression correlated with hallmarks of disease progression, including poor survival ( $P = 0.01$ , Spearman correlation), recurrence ( $P = 6 \times 10^{-5}$ , Spearman correlation), and advanced Gleason grade ( $P = 0.0004$ ) in prostate cancer, N-myc amplification in neuroblastoma, *Helicobacter pylori* infection in gastric cancer, and chromosome 11q13 amplification in head and neck cancer (Supplementary Table S2).

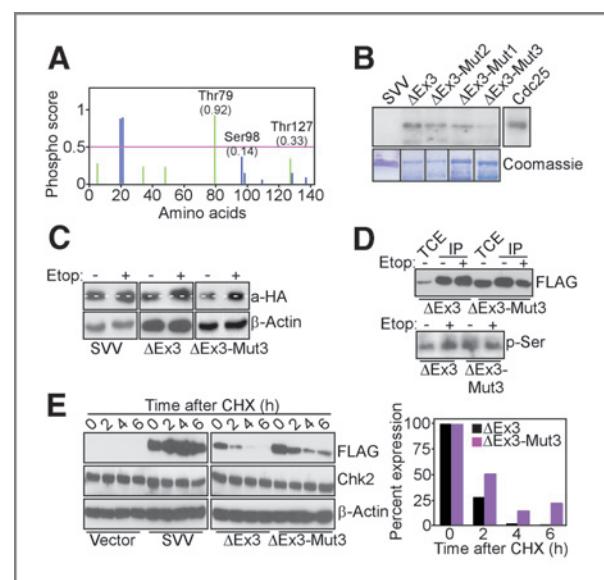
To validate these bioinformatics predictions, quantitative PCR analysis of primary tumor samples and matched normal tissues was next carried out. In these experiments, survivin- $\Delta$ Ex3 mRNA was consistently overexpressed in cases of non-small cell lung cancer (Fig. 1E, left) and prostate cancer (Fig. 1E, right), compared with matched normal tissues.

### Subcellular localization of survivin- $\Delta$ Ex3

Consistent with previous observations (6, 7), endogenous survivin- $\Delta$ Ex3 mRNA was expressed at lower abundance in MCF-7 cells, compared with WT survivin (Supplementary Fig. S2A). When transfected in HCT116, H460, or MDA-431 tumor cells, FLAG-tagged survivin- $\Delta$ Ex3 was exclusively localized to nuclei, whereas WT survivin was distributed in nuclear and cytosolic fractions (Fig. 2A; ref. 23). In addition, survivin- $\Delta$ Ex3 did not localize to mitochondria in transfected tumor cells, as opposed to an abundant pool of WT survivin associated with tumor mitochondria (Fig. 2B). Functionally, treatment of H460 cells with the apoptosis inducer, staurosporine, caused loss of



**Figure 2.** Nuclear localization of survivin- $\Delta$ Ex3 in cancer. A, the indicated tumor cells were transfected with vector, FLAG-WT survivin (SVV) or FLAG-survivin- $\Delta$ Ex3 and cytosolic (C) or nuclear (N) fractions were analyzed by Western blotting. RCC1 was a nuclear marker. B, the indicated tumor cells were transfected as in A, and cytosolic (C) or mitochondrial (M) fractions were analyzed by Western blotting. COX-IV was a mitochondrial marker. For A and B, the position of survivin (top) or survivin- $\Delta$ Ex3 (bottom) bands is indicated. C, MDA-431 cells were transfected with vector or survivin- $\Delta$ Ex3, treated with staurosporine (STS), and analyzed for mitochondrial membrane potential by JC-1 staining and multiparametric flow cytometry. Data are expressed as ratio between green (FL1)/red (FL2) fluorescence. The percentage of cells in each quadrant is indicated.



**Figure 3.** Chk2 phosphorylation of survivin- $\Delta$ Ex3. A, the position of 3 putative Chk2 phosphorylation sites at Thr79, Ser98, and Thr127 in survivin- $\Delta$ Ex3 is indicated. B, active Chk2 was incubated with recombinant survivin (SVV), survivin- $\Delta$ Ex3, or survivin- $\Delta$ Ex3 Thr127—Ala (Mut1), Thr79/Ser98—Ala (Mut2), or Thr127/Thr79/Ser98—Ala (Mut3) in a kinase assay, and radioactive bands were visualized by autoradiography. Cdc25 was a control Chk2 substrate. Bottom, Coomassie blue staining of recombinant proteins. C, HCT116 cells were transfected with the indicated FLAG-tagged cDNA constructs, treated with (+) or without (-) etoposide and analyzed for changes in electrophoretic migration of phospho-tagged protein bands, by Western blotting. A white dotted line is drawn across the center of each band. D, HCT116 transfected with FLAG-survivin- $\Delta$ Ex3 or FLAG-survivin- $\Delta$ Ex3-Mut3 were immunoprecipitated (IP) with an antibody to FLAG before or after etoposide treatment (top), and immune complexes were analyzed with an antibody to phosphorylated Ser residues by Western blotting (bottom). TCE, total cell extracts. E, HCT116 cells transfected as indicated were treated with cycloheximide (CHX), harvested at the indicated time intervals after release, and analyzed by Western blotting. Right, densitometric quantification of protein bands. Similar results were obtained in an independent experiment: survivin- $\Delta$ Ex3, 2 hours, 61.2%; 4 hours, 40.6%; 6 hours, 3.6%; survivin- $\Delta$ Ex3-Mut3, 2 hours 58.1%; 4 hours, 48.7; 6 hours, 25.7%.

mitochondrial transmembrane potential, in a reaction unaffected by transfection of control plasmid or survivin- $\Delta$ Ex3 (Fig. 2C). Conversely, a ~50% knockdown of endogenous survivin- $\Delta$ Ex3 expression using isoform-specific siRNA (Supplementary Fig. S2A) produced nuclear events, with accumulation of tumor cells with G2/M DNA content, and appearance of a cell population with >4N DNA content (Supplementary Fig. S2B). In contrast, survivin- $\Delta$ Ex3-specific siRNA did not reduce WT survivin mRNA levels, and a nontargeting siRNA had no effect (Supplementary Fig. S2A and S2B).

### Chk2 phosphorylation of survivin- $\Delta$ Ex3

By sequence analysis, 3 potential phosphorylation sites matching the consensus for the DNA damage kinase, Chk2 (16) were identified at Thr79, Ser98, and Thr127 in the unique -COOH terminus of survivin- $\Delta$ Ex3 (Fig. 3A; refs. 6,

7). In contrast, no Chk2 phosphorylation sites were predicted in WT survivin. Accordingly, active Chk2 readily phosphorylated recombinant survivin-ΔEx3, but not WT survivin, in a kinase assay (Fig. 3B). Ala substitution of Thr127 in survivin-ΔEx3 (ΔEx3-Mut1), or double mutation of Thr79 and Ser98 (ΔEx3-Mut2) partially reduced phosphorylation by Chk2 (Fig. 3B). In addition, Ala mutagenesis of all 3 predicted Chk2 phosphorylation sites at Thr127, Thr79, and Ser98 (ΔEx3-Mut3) abolished Chk2 phosphorylation of survivin-ΔEx3 (Fig. 3B).

We next asked whether Chk2 phosphorylated survivin-ΔEx3, *in vivo*, potentially as part of a DNA damage response in tumor cells (17). First, treatment of HCT116 cells with the DNA damaging agent, etoposide, did not result in electrophoretic mobility changes of transfected FLAG-WT survivin (Fig. 3C). In contrast, etoposide-induced DNA damage caused gel retardation of FLAG-survivin-ΔEx3 in HCT116 transfectants (Fig. 3C). A Chk2 phosphorylation-defective FLAG-survivin-ΔEx3-Mut3 (Fig. 3B) transfected in HCT116 cells did not exhibit gel shift retardation after DNA damage (Fig. 3C), and no changes in electrophoretic mobility were observed for survivin-ΔEx3 or survivin-ΔEx3-Mut3 in untreated cultures (Fig. 3C). Second, survivin-ΔEx3 immune complexes precipitated from etoposide-treated HCT116 cells (Fig. 3D, top) reacted with an antibody to phosphorylated Ser residues, compared with untreated cultures (Fig. 3D, bottom). In contrast, survivin-ΔEx3-Mut3 immunoprecipitates (Fig. 3D, top) did not exhibit changes in phosphorylated Ser reactivity, with or without etoposide (Fig. 3D, bottom).

A potential regulation of survivin-ΔEx3 by Chk2 phosphorylation was next investigated. WT survivin transfected in HCT116 cells remained stable over a 6-hour period (Fig. 3E). In contrast, survivin-ΔEx3 exhibited a much accelerated turnover, with an estimated half-life of ~1 hour (Fig. 3E). Ablation of Chk2 phosphorylation in survivin-ΔEx3-Mut3 improved protein stability, prolonging its half-life to ~2 hours (Fig. 3E). Expression of survivin-ΔEx3 or survivin-ΔEx3-Mut3 did not affect endogenous Chk2 levels in HCT116 transfectants (Fig. 3E). In addition, recombinant survivin-ΔEx3 or survivin-ΔEx3-Mut3 did not associate with endogenous Chk2 in pull down experiments (Supplementary Fig. S3A). As control, recombinant survivin, but not survivin-ΔEx3, associated with the mitochondrial chaperone, Hsp60 in tumor cells (Supplementary Fig. 3B; ref. 19).

#### **Participation of survivin-ΔEx3 in a DNA damage-sensing checkpoint in tumor cells**

Etoposide treatment of HCT116 cells resulted in the appearance of punctate nuclear foci containing Ser139-phosphorylated histone H2AX or γH2AX (24), a marker of unrepaired DNA damage (17), by fluorescence microscopy (Fig. 4A and B). Expression of WT survivin or survivin-ΔEx3 did not affect formation of γH2AX-containing foci in response to etoposide (Fig. 4A and B). In contrast, transfection of survivin-ΔEx3-Mut3 inhibited the appearance of γH2AX-containing nuclear foci after DNA damage (Fig. 4A and B). Similar results were obtained by quantification of γH2AX reactivity by multiparametric flow cytometry. Accordingly, HCT116 cells transfected

with vector, WT survivin, or survivin-ΔEx3 exhibited increased γH2AX reactivity after DNA damage, in a response that peaked 16 hours after etoposide treatment (Supplementary Fig. S4A), and returned to baseline during a 4- to 8-hour recovery period (Fig. 4C). In contrast, transfection of survivin-ΔEx3-Mut3 suppressed the induction of γH2AX reactivity after DNA damage (Fig. 4C; Supplementary Fig. S4A).

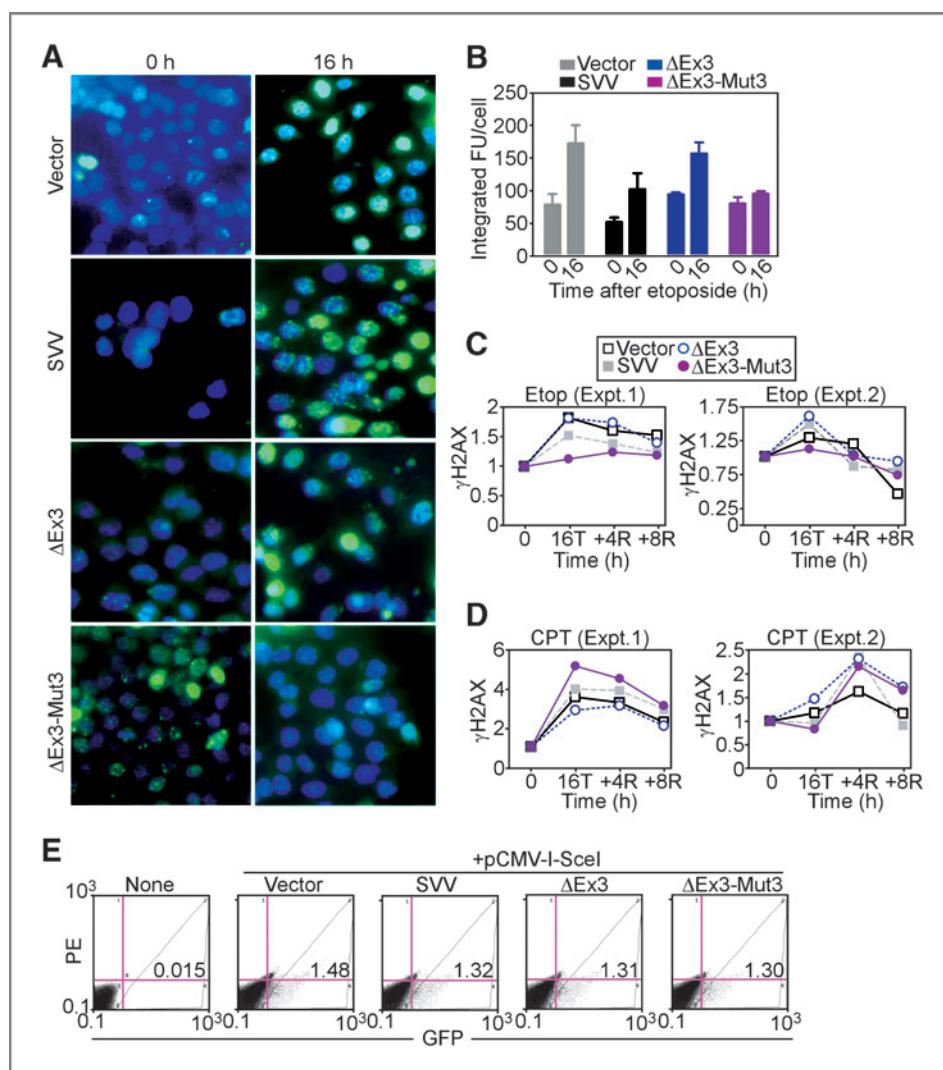
To test the specificity of γH2AX modulation by survivin-ΔEx3, we next treated HCT116 cells with the topoisomerase I inhibitor, camptothecin (CPT), which induces single-strand DNA breaks, as opposed to the double-strand breaks (DSB) caused by etoposide. CPT induced robust and time-dependent γH2AX reactivity in vector transfectants, by multiparametric flow cytometry (Fig. 4D; Supplementary Fig. S4B). Similarly, transfection with WT survivin, survivin-ΔEx3, or survivin-ΔEx3-Mut3 did not affect the magnitude or time-course of γH2AX induction by CPT (Fig. 4D; Supplementary Fig. S4B).

We next asked whether Chk2 phosphorylation of survivin-ΔEx3 affected homologous-directed DSB repair using HCT116 cells stably transfected with a GFP-DR recombination reporter (21, 22). Cotransfection of HCT-pDR-GFP cells with pCMV-I-SceI plus vector resulted in the appearance of GFP<sup>+</sup> cells, indicative of homologous DSB repair (Fig. 4E; refs. 21, 22). Addition of WT survivin, survivin-ΔEx3, or survivin-ΔEx3-Mut3 to these cells also resulted in quantitatively comparable degree of homologous DSB repair (Fig. 4E). No GFP<sup>+</sup> cells were detected in the absence of pCMV-I-SceI (Fig. 4E), ruling out spontaneous intrachromosomal DNA conversion (21, 22).

#### **Requirements of survivin-ΔEx3 for a DNA damage-sensing checkpoint**

We next asked whether the effect of survivin-ΔEx3 on DSB foci formation reflected changes in total H2AX expression. HCT116 cells transfected with survivin-ΔEx3 or survivin-ΔEx3-Mut3 did not exhibit significant changes in endogenous H2AX levels, in the presence or absence of DNA damage (Fig. 5A). Second, we tested whether this pathway required the DNA damage-sensing Mre11-Rad50-Nsb1 (MRN) complex, which is deficient in HCT116 cells (25). Transfection of survivin-ΔEx3 in colorectal adenocarcinoma SW480 cells, which express the MRN complex, resulted in robust γH2AX induction in response to etoposide, and this response was abolished by expression of survivin-ΔEx3-Mut3 (Fig. 5B).

Finally, we looked at the activation of downstream pathway(s) of DNA damage upon expression of the various survivin isoforms. Transfection of survivin-ΔEx3-Mut3 suppressed γH2AX induction in etoposide-treated HCT116 cells (Fig. 5C), consistent with the data above. However, stabilization of p53, phosphorylation of p53 on Ser15, or upregulation of the cyclin-dependent kinase inhibitor, p21, was indistinguishable in cells expressing survivin-ΔEx3 or survivin-ΔEx3-Mut3 (Fig. 5C). Similarly, no differences were observed in etoposide-induced Thr68 phosphorylation of Chk2 in the presence of the various survivin isoforms (Fig. 5C), and transfection of survivin-ΔEx3 or survivin-ΔEx3-Mut3 did not alter the expression of the chromosomal passenger protein, Aurora B during DNA damage, compared with WT survivin (Supplementary Fig. S5).



**Figure 4.** Survivin-ΔEx3 regulation of a DNA damage-sensing checkpoint. **A**, HCT116 cells were transfected with the indicated cDNAs, treated with etoposide, and analyzed at the indicated time intervals for  $\gamma$ H2AX-containing foci formation by fluorescence microscopy; magnification,  $\times 100$ . **B**, quantification of  $\gamma$ H2AX foci formation. FU, fluorescence units. **C** and **D**, HCT116 cells were transfected as indicated, exposed to etoposide (Etop; **C**) or camptothecin (CPT; **D**) for a 16-hour treatment (16T), followed by 4- and 8-hour recovery (+4R, +8R) and analyzed for  $\gamma$ H2AX expression by multiparametric flow cytometry. Normalized data from 2 independent experiments (Expt) are shown. **E**, HCT116 DR-GFP cells were transfected with pCMV-I-SceI without (None) or with the indicated cDNAs, and analyzed for changes in GFP expression, by multiparametric flow cytometry. The percentage of GFP<sup>+</sup> cells under the various conditions tested is indicated. PE, phycoerythrin.

### Cellular implications for survivin-ΔEx3 participation in the DNA damage response

In the absence of DNA damage, transfection of survivin-ΔEx3 or survivin-ΔEx3-Mut3 significantly enhanced HCT116 colony formation in soft agar, compared with control cultures (Fig. 5D, left), suggesting a cytoprotective role of both molecules irrespective of DNA damage or Chk2 phosphorylation. Expression of survivin-ΔEx3 still supported HCT116 colony formation after etoposide-induced DNA damage (Fig. 5D, right). In contrast, transfection of survivin-ΔEx3-Mut3 impaired the ability of HCT116 cells to form large colonies (up to 200  $\mu$ m in diameter) after etoposide treatment (Fig. 5D, right).

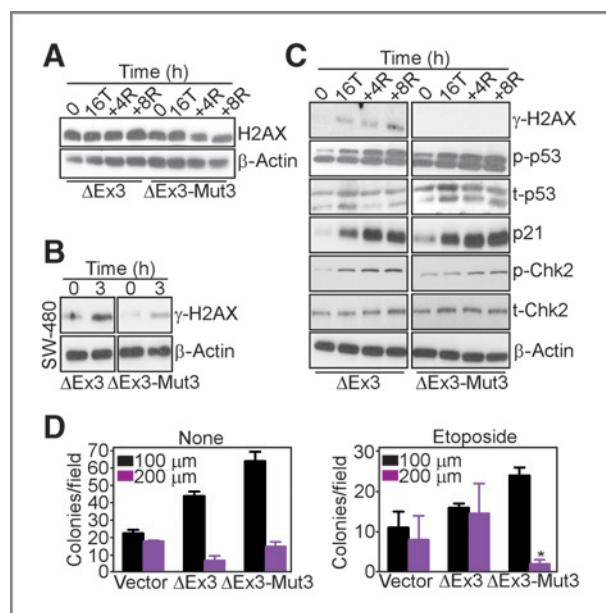
As far as potential implications of this pathway for human tumorigenesis, Thr68-phosphorylated, that is active Chk2 became abundantly expressed at the earliest, hyperplastic stages of the colorectal adenoma-to-carcinoma transition, and persisted in advanced disease stages (Supplementary Fig. S6A and S6B). In contrast, active Chk2 was undetectable in normal epithelium, and total Chk2 levels remained unchanged

throughout disease progression (Supplementary Fig. S6A and S6B). The expression of active Chk2 correlated with aberrantly increased levels of survivin and p53, which rose in dysplastic lesions and remained elevated throughout all stages of colorectal transformation (Supplementary Fig. S6A and S6B).

### Discussion

In this study, we have shown that survivin-ΔEx3 (6, 7) is a nuclear protein phosphorylated by the checkpoint kinase, Chk2 (16) during DNA damage on 3 novel amino acids in its unique -COOH terminus. Ablation of Chk2 phosphorylation enhanced survivin-ΔEx3 stability *in vivo*, suppressed  $\gamma$ H2AX reactivity specifically in response to DSB, and impaired long-term clonogenic tumor cell survival. When analyzed in human tumors, survivin-ΔEx3 was differentially expressed in tumors compared with normal tissues, correlating with markers of unfavorable prognosis.

Previous studies had suggested a link between survivin and the DNA damage response. This was originally associated with



**Figure 5.** Requirements of survivin-ΔEx3 during the DNA damage response. **A**, HCT116 cells were transfected as indicated, incubated with etoposide for a 16-hour treatment (16T) followed by 4- and 8-hour recovery (+4R, +8R), and analyzed by Western blotting. **B**, SW480 cells transfected and treated as in A were analyzed by Western blotting. **C**, transfected HCT116 cells were treated as in A and analyzed by Western blotting. **D**, HCT116 cells were transfected as indicated, left untreated (None) or exposed to etoposide, and analyzed for colony formation in semisolid medium. Colonies were imaged by phase contrast microscopy (magnification,  $\times 50$ ), and quantified for diameters of 50 to 100  $\mu$ m or 100 to 200  $\mu$ m. Mean  $\pm$  SEM of replicates from 2 independent experiments.

the ability of survivin to counter radiation-induced apoptosis (26), but recent evidence suggested a more direct participation of the survivin network in DNA damage and repair, potentially independent of cytoprotection (27). Accordingly, interference with survivin expression or function resulted in reduced DNA repair, especially in gliomas (28), and therapeutic targeting of survivin with the small molecule transcriptional suppressor, YM155 (29), delayed DSB repair (30). Despite a potential suggestion that survivin may control the expression of the DNA repair protein, Ku70 (31), the molecular basis for a potential *direct* participation of the survivin network in DNA damage or repair has remained largely unexplored.

The data presented here add a new layer of complexity to these observations, and propose a role of the survivin spliced variant, ΔEx3 (6, 7) in *sensing* unrepaired DNA damage in tumor cells. This extends the panoply of functions previously associated with survivin-ΔEx3, including nucleolar (32), or mitochondrial control of apoptosis inhibition (33, 34), or endothelial cell migration and invasion (11), implicating Chk2 phosphorylation (16) as a novel regulator of survivin-ΔEx3. As shown here, Chk2 phosphorylation of survivin-ΔEx3 targeted its unique -COOH terminus introduced by the splicing event (6, 7), occurred during DNA damage, *in vivo*, and contributed to accelerated degradation of survivin-ΔEx3, compared with WT survivin. A role of survivin-ΔEx3 in the DNA damage response

had not been previously proposed; however, this is consistent with the exclusive nuclear localization of this survivin isoform (35), which has been postulated to involve a bipartite nuclear localization signal, also introduced in its -COOH terminus by the splicing event (36).

Against this backdrop, Chk2 has long been recognized as a pivotal regulator of genomic integrity in response to DNA damage. Activated via phosphorylation on Thr68 by the DNA damage sensor kinase, ataxia telangiectasia mutated (ATM), active Chk2 in turn phosphorylates panoply of downstream targets coordinating checkpoint activation, cell-cycle arrest, and apoptosis (16). Adding to this scenario, Chk2 phosphorylation of survivin-ΔEx3 was required to induce time-dependent induction of γH2AX, a marker of unrepaired DNA damage (24) important to recruit repair proteins to nuclear foci (37). Further studies are required to fully elucidate how a Chk2-survivin-ΔEx3 signaling axis contributes to a DNA damage response in tumor cells. However, the evidence presented here suggests that this pathway is specific for DSB, does not affect endogenous H2AX levels, is independent of the MRN complex (38), and is not required for homologous-directed DNA repair. Taken together, these findings suggest a model in which Chk2 phosphorylation of survivin-ΔEx3 provides a feedback amplification loop to enhance DSB *sensing* via optimal induction of γH2AX, which, in turn, may facilitate recruitment of repair proteins, including DNA-PK (39) or Ku70 (31), at sites of DNA damage (27). Given the rapid turnover of Chk2-phosphorylated survivin-ΔEx3, *in vivo*, this pathway may contribute to the earliest assembly of the repair machinery at DNA foci, and would be expected to confer a survival advantage for tumor cells exposed to genotoxic stimuli. Consistent with this hypothesis, survivin-ΔEx3 was preferentially observed in tumors compared with normal tissues, and correlated with markers of aggressive disease, *in vivo* (40, 41). Conversely, loss of Chk2 phosphorylation of survivin-ΔEx3 was detrimental for tumor growth, and impaired long-term clonogenic tumor cell survival after DSB.

Aside from its tumor suppressive properties, which are inactivated by mutagenesis in selected tumors (42), Chk2 has been intensely pursued for novel cancer therapeutics (43). Broadly defined as "checkpoint abrogation," this strategy relies on the premise that pharmacologic inhibition of Chk2 (and Chk1) may prevent cell cycle arrest in response to DNA damage, and thus sensitize tumor cells to apoptosis (44). While validating Chk2 as an important cancer therapeutic target (45), the data presented also anticipate a broader role of this pathway in tumor progression, via exploitation of the survivin network. Mechanistically, this may involve Chk2-mediated release of cytoprotective survivin from its mitochondrial stores during DNA damage (18), coupled with efficient DSB *sensing* via phosphorylation of survivin-ΔEx3 (this study). More evidence is needed to further credential this model, *in vivo*, but it is intriguing that both active Chk2 and survivin become expressed at the earliest stages of malignant transformation in humans, for instance during the adenoma-to-carcinoma transition. The early appearance of Chk2 in comparable settings has been interpreted as a barrier to further malignant transformation (46, 47). However, it is also possible that Chk2

exploitation of the survivin network under these conditions may paradoxically contribute to accelerated tumor development, simultaneously promoting resistance to apoptosis (18), and faster control of genotoxic damage (this study). This scenario also reiterates the broad contribution of survivin family proteins to multiple aspects of tumorigenesis (1), and validate therapeutic strategies aimed at *globally* suppressing aberrant *survivin* gene transcription in tumors (30).

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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