Journal of Cellular Biochemistry 100:217–229 (2007)

Functional Analysis of Survivin in Spindle Assembly in *Xenopus* Egg Extracts

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Abstract Survivin is a member of the inhibitor of apoptosis (IAP) protein family that serves critical roles in mitosis and cytokinesis. Many studies have suggested Survivin's involvement in spindle regulation, but direct biochemical evidence for this has been lacking. Using the cell-free system of *Xenopus* egg extracts, we tested whether Survivin was necessary for the assembly of metaphase spindles. Removal or inhibition of *Xenopus* Survivin causes the disruption in the formation of metaphase spindles. In particular, we observe the generation of microtubule (MT) asters or poorly formed shortened spindle structures. In the latter phenotype the spindle structures display a decrease pole-to-pole length and a reduction of MTs around the chromatin indicating that Survivin may promote the stabilization of MT–chromatin interactions. In addition, function analysis of Survivin's conserved phosphorylation site Thr34 (Thr43 in *Xenopus*) and tubulin-binding domain was also assessed in regulating spindle assembly. Treatment of *Xenopus* egg extracts with a recombinant Survivin mutant that contained an alanine residue substitution at Thr43 (SURT43A mutant) or that was missing the C-terminal tubulin-binding domain (SURCL mutant) produced an increased frequency of MT asters and shorten abnormal spindle structures in *Xenopus* egg extracts. Interestingly, a phosphomimetic mutation made at residue Thr43 of Survivin (SURT43E mutant) generated a high frequency of MT asters implying that premature 'activation' of Survivin may interfere with an early stage of spindle assembly. Taken together, we propose that Survivin is a necessary component of the mitotic spindle and its phosphorylation at residue Thr43 is important for Survivin function in spindle assembly. J. Cell. Biochem. 100: 217–229, 2007.

Key words: Survivin; spindle assembly; xenopus; phosphorylation; MT aster

Survivin is an essential regulator of mitosis. As a member of the inhibitor of apoptosis (IAP)[1] gene family, Survivin contains a single baculovirus IAP repeat (BIR) and an extended COOH terminus α-helical coiled-coil domain [Ambrosini et al., 1997]. The COOH terminus α-helical domain of Survivin is important for dimerization [Muchmore et al., 2000] as well as for binding to polymerized tubulin [Li et al., 1998]. Survivin expression is restricted to G_2/M phases of the cell-cycle [Kobayashi et al., 1999; Li et al., 1999] where upon it localizes to various components of the mitotic apparatus. During late prophase Survivin becomes localized at the centrosomes, moves to the spindle pole microtubules (MT) and kinetochores at metaphase, re-localizes to the spindle midzone at anaphase, and concentrates at the midbody during cytokinesis [Li et al., 1998; Skoufias et al., 2000; Fortugno et al., 2002]. Thus, the temporal and spatial pattern of Survivin expression is consistent with the proposal that Survivin contributes to regulating multiple phases of cell division.

This article contains supplementary material, which may be viewed at the Journal of Cellular Biochemistry website at http://www.interscience.wiley.com/jpages/0730-2312/suppmat/index.html.

Abbreviations used: IAP, inhibitor of apoptosis; BIR, baculovirus IAP repeat; Thr, threonine; Cdk1, cyclin-dependent kinase 1; INCENP, inner centromere protein; GST, glutathione-*S*-transferase; His, histidine; IPTG, isopropyl-1-thio-β-D-galactopyranoside; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; CSF, cytostatic factor; PBS, phosphate-buffer saline; MT, microtubule

Grant sponsor: National Institutes of Health; Grant number: NIH GM62542.

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Received 26 January 2006; Accepted 9 June 2006

DOI 10.1002/jcb.21065

Loss of function studies have implicated Survivin in chromosome segregation [Li et al., 1999; Uren et al., 2000; Leverson et al., 2002; Lens et al., 2003; Yang et al., 2004], the spindle checkpoint arrest [Li et al., 1998; Lens et al., 2003; Petersen and Hagan, 2003], spindle elongation [Uren et al., 1999; Morishita et al., 2001], and cytokinesis [Fraser et al., 1999; Li et al., 1999; Speliotes et al., 2000; Yang et al., 2004]. Disruption of Survivin's genetic locus leads to an embryonic lethal phenotype in homozygous mice further supporting its essential role in mitosis [Uren et al., 2000]. Phosphorylation of Survivin at its conserved threonine (Thr)34 residue by cyclin-dependent kinase 1 (Cdk1) [O'Connor et al., 2000] or Thr117 residue by Aurora B [Wheatley et al., 2004] is important for Survivin function and localization to the spindle apparatus. Finally, a recent study shows that regulation of Survivin ubiquitination controls both the dynamic association of Survivin with the centromeres and the proper targeting of Survivin and Aurora B to centromeres [Vong et al., 2005]. Collectively, a picture has emerged linking the involvement of Survivin to the regulation of the mitotic spindle. However, it is currently unclear whether Survivin functions occur prior to metaphase in regulating the formation of the bipolar spindle.

In this study we use *Xenopus* egg extracts to test whether Survivin has a direct role in regulating spindle assembly. Several approaches were employed to interfere with Survivin function including antibody interference, immunodepletion, and the addition of recombinant Survivin mutant proteins. All approaches caused a block in normal spindle assembly in *Xenopus* egg extracts. The abnormal spindle structures generated include MT asters or shortened spindle structures that contain reduced MTs around the mitotic chromatin. We propose that Survivin is critical for spindle assembly and participates in the stabilization of chromatin-associated MTs.

MATERIALS AND METHODS

Cloning and Mutagenesis of *Xenopus* Survivin

A DNA clone from a *Xenopus* cDNA library at Washington University was obtained based on sequence homology to Survivin. DNA sequencing of the cDNA clone (performed by the Moffitt Cancer Center molecular biology facility) established an open reading frame encoding a 160 amino acid protein with strong homology to mouse and human Survivin (see supplementary Fig. S1) that is identical to a previously described *Xenopus* Survivin cDNA clone [Bolton et al., 2002]. The *Xenopus* Survivin full-length cDNA was amplified by PCR and subcloned into an *EcoRI-NotI* cut pGEX$(His)_6$ vector to allow for the expression of glutathione-*S*-transferase (GST)-tagged Survivin$(His)_6$ protein. pGEX $(His)_6$ is a modified version of pGEX4T-3 (Amersham Pharmacia) in which a histidine $(His)_6$-tag insert was subcloned into a *NotI* site in the multicloning region. *Xenopus* Survivin Thr43 to alanine (T43A) and Thr43 to glutamate (T43E) mutations were introduced into the wild-type pGST-Survivin$(His)_6$ vector using the GeneEditor site-directed mutagenesis system (Promega). To prepare a deletion mutant missing the COOH terminal alpha heli extended domain of Survivin, amino acids 1–109 of Survivin were PCR-amplified (SURCL) and subcloned into pGEX$(His)_6$. DNA sequencing by the Moffitt sequencing facility confirmed the nucleotide sequence of the T43A and T43E mutations and the SURCL deletion mutant.

Purification of Recombinant Proteins From *E. coli*

Recombinant GST-Survivin$(His)_6$ proteins were expressed in *E. coli* strain BL21 (DE3) by inducing for 1.5 h with 0.2 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at 30°C. Bacteria cells were pelleted by centrifugation and stored at −80°C. Recombinant proteins were purified using Ni^{2+}-chelate resin affinity chromatography (Novagen) followed by glutathione sepharose chromatography (Amersham Pharmacia) using manufacturer suggested protocols with some modifications. Briefly, thawed bacteria pellets were resuspended in Binding buffer (20 mM Tris-HCl, pH 7.9, 500 mM NaCl, and 5 mM imidazole) containing protease inhibitors (100 μM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml each of leupeptin, pepstatin A, and aprotinin), and sonicated 4× 15 s on ice. Bacteria lysates were supplemented with 1% Triton X-100 and incubated for 30 min at 4°C. The lysates were clarified by centrifugation, filtered through a 0.45 μm filter and incubated with Ni^{2+}-chelate resin batch-wise for 1 h at 4°C with rotation to bind C-terminal $(His)_6$-tagged proteins. Ni^{2+}-chelate resin complexes were loaded onto Biorad econo columns and washed with 10 volumes of

Binding buffer followed by 6 volumes of Binding buffer containing 60 mM imidazole. Binding buffer containing 500 mM imidazole was used to elute $(His)_6$-tagged proteins. Eluted fractions containing high concentrations of protein were pooled and diluted fourfold with 20 mM Tris-HCl, pH 7.9, and 500 mM NaCl to reduce imidazole concentration. Triton X-100 (1% final) and dithiothreitol (DTT) (10 mM final) were added to the diluted samples and mixed with glutathione Sepharose beads for 30 min at 4°C with rotation. Glutathione bead complexes were washed with 20 mM Tris-HCl, pH 7.9, 500 mM NaCl and 10 mM DTT, and GST-tagged proteins were eluted in 20 mM glutathione, 10 mM HEPES, pH 8.0, and 10 mM DTT. Eluted fractions containing GST-Survivin $(His)_6$ protein were concentrated using Centricon-10 concentrators (Amicon Bioseparations) and buffer exchanged into 10 mM HEPES, pH 8.0. Finally, 10% (v/v) glycerol was added to the protein preparation before storage into small aliquots at −80°C. This two-step affinity purification protocol allowed for the isolation of full-length recombinant GST-Survivin$(His)_6$ with a purity of ~90% as assessed by coomassie stained gels. Final concentration of GST-Survivin$(His)_6$ proteins fell within the range of 0.5–1.0 μg/μl; this was estimated by separating 1-μl aliquots of sample preparation on SDS–PAGE, staining with commassie blue, and comparing the stained protein band with known amounts of bovine serum standards.

Production, Purification, and Characterization of Xenopus Survivin Antibodies

A GST fusion to full-length Xenopus Survivin protein was used to immunize two rabbits for the production of polyclonal Survivin antibodies (Zymed laboratories, South San Francisco, CA). Sera from rabbit #2, but not pre-immune sera, readily recognized thrombin-cleaved recombinant Xenopus Survivin protein (as low as 1–2 ng) by Western analysis. Anti-Survivin antibodies were partially purified by passing the rabbit serum over a GST column several times to remove contaminating anti-GST antibodies. The flow through fraction was then passed over a protein A agarose column to isolate the remaining IgG fraction. Bound antibodies were eluted with 100 mM acetic acid and immediately neutralized in 1.5 ml tubes containing 100 μL of 1 M Tris-HCl, pH 8.0. Survivin antibodies were dialyzed in 10 mM HEPES, pH 7.2, and 100 mM KCl, and stored at −80°C. The partially purified Survivin antibodies immunoprecipitate an 18–20 kDa protein from Xenopus egg extracts not detected with pre-immune sera (supplementary Fig. S2). In addition, immunoprecipitated Survivin complexes from Xenopus egg extracts show that Cdk1 (also called Cdc2) protein is co-precipitated at mitosis (supplementary Fig. S3), which is consistent with earlier reports in human cells [O'Connor et al., 2000]. A comparison of immunoblot signals of Survivin from 1 μl of Xenopus egg extract to known amounts of thrombin cleaved recombinant Survivin allowed us to estimate the concentration of Survivin to be ~30 nM (data not shown).

Preparation of Xenopus Egg Extracts and Spindle Assembly

Preparation of cytostatic factor (CSF)-arrested egg extracts and spindle assembly reactions were performed essentially as described [Desai et al., 1999]. Metaphase spindles were typically assembled in cycled CSF extracts, except for immunodepletion experiments in which CSF-arrested extracts were used. To monitor MTs of spindles, rhodamine-labeled bovine brain tubulin (Cytoskeleton, Denver, CO) was added to a final concentration of 0.1 μg/μl in extracts. Spindles and associated chromosomes were visualized by applying 2 μl of extract and 6 μl of fixative (60% glycerol (v/v), 0.l mM HEPES, pH 7.5, 10% formaldehyde, and 1 μg/ml bisbenzimide) to a microscope slide and examined by epifluorescence microscopy.

Antibody Interference and Immunodepletions

For antibody interference experiments, antibodies dialyzed in 10 mM HEPES, pH 7.2, and 100 mM KCl were used for adding to extracts. As a control for the specificity of the Survivin antibody, GST-Survivin fusion protein (2.5 μg) was pre-incubated with partially purified *Xenopus* Survivin antibody for 30 min at room temperature prior to adding to *Xenopus* egg extracts for testing spindle assembly. For immunodepletion of Survivin from CSF-arrested extracts, anti-Survivin antibodies were pre-bound to protein A-sepharose 4B fast-flow beads (Sigma) for 2 h at 4°C. The antibody bead complexes were washed and equilibrated in XB buffer (10 mM HEPES-KOH, pH 7.7, 50 mM sucrose, 100 mM KCl, 0.1 mM $CaCl_2$, and 1 mM $MgCl_2$). Next

CSF-arrested extracts were incubated with antibody-bead complexes (5–10 μl CSF extract/μl beads) for 45–60 min at 4°C with rotation. Egg extracts were subjected to a second round of depletion to allow for the removal of 90% or greater endogenous Survivin. In parallel, mock depletions were carried out using rabbit IgG. Both mock- and Survivin-depleted extracts were used for spindle assembly reactions. To analyze Survivin protein bound to the beads, antibody-bead complexes were washed 3–4× with XB buffer, boiled in SDS sample buffer, and subjected to SDS–PAGE and immunoblot analysis.

Chromatin Isolation and Pelleting of Microtubules

Purification of chromatin from *Xenopus* egg extracts and the analysis of associated proteins by Western analysis were carried out essentially as described [Arnaoutov and Dasso, 2003]. Briefly, 25 μl of CSF-arrested or cycled egg extracts (containing spindle/chromatin structures) was diluted 1:10 in buffer B solution (10 mM HEPES-KOH [pH 7.7], 50 mM KCl, 2 mM $MgCl_2$, 5 mM EGTA, and 40 mM β-glycerophosphate) containing 15% glycerol and 10 μg/ml each of leupeptin, pepstatin, and aprotinin. The diluted extract was incubated for 1 min at room temperature and then layered onto a 40% glycerol cushion in buffer B and immediately centrifuged for 5 min at 4°C at 20,000*g*. The chromatin pellet was gently washed in buffer B containing 1% triton X-100 and centrifuged again at the same conditions as described above. Finally, the chromatin pellet was resuspended in 25 μl SDS-sample buffer, boiled for 5 min, and separated by SDS–PAGE followed by Western analysis.

To assess the binding of recombinant Survivin mutant proteins to MTs in *Xenopus* egg extracts, 25 μl aliquots of extract (containing 300 nM final of wild-type or mutant Survivin) were diluted with 500 μl 1× BRB80 (80 mM Pipes-KCl, pH 6.8, 1 mM $MgCl_2$, and 1 mM EGTA)/30% glycerol and layered onto 1 ml 40% glycerol cushions in 1× BRB80. Samples were centrifuged for 20 min at maximum speed in a microfuge at 4°C. MT pellets were gently washed with 200 μl 1× BRB80/1% Triton X-100, centrifuged again and then solubilized in SDS sample buffer by heating at 95°C for 5 min. Immunoblot analysis was performed to detect the presence of recombinant Survivin protein associated with the insoluble tubulin fraction.

Immunoblot Analysis

For Western analysis, membranes were incubated in 5% non-fat dry milk/1× phosphate-buffer saline (PBS) containing 0.2% Tween 20 for 30 min. All primary antibodies were used at a 1:1,000 dilution in blocking solution: anti-Survivin, anti-cdc2 mouse monoclonal (clone Ab-3, Calbiochem), anti-α-tubulin mouse monoclonal (clone B-5-1-2; Sigma), anti-His tag rabbit polyclonal (H15, Santa Cruz), and anti-phospho-H3 (Upstate Biotechnology). Membranes were washed three times (10 min each) with 1× PBS containing 0.2% Tween-20. Rabbit (Sigma) or Mouse (Jackson Laboratories) IgG antibodies conjugated to alkaline phosphatase were used as secondary antibodies (1:2,500–1:5,000 dilution range). Proteins were detected on blots by incubating with the CDP-star chemiluminescence substrate (Roche Diagnostic) for 5 min at room temperature and exposing to Kodak X-ray film.

Fluorescence Imaging and Quantitative Analysis

Spindle images were captured with a Roper coolsnap HQ CCD camera mounted to a Nikon E800 fluorescence microscope and controlled by the Metamorph software v5.0r1 (Universal Imaging Corp.). Image processing of spindle structures was performed using Metamorph and Adobe Photoshop v.7 software. Metamorph software was used to measure the pole-to-pole length of spindles in microns. To determine MT density from pole-to-pole, tubulin fluorescence along a straight line of the spindle was measured by ImageQuant software, and then exported into Microsoft Excel and graphed for analysis.

Statistics

Data in graphs are reported as mean ± SD and the significance of difference between means was assessed using student's *t*-test. $P < 0.05$ and $P < 0.01$ was considered to be significant and highly significant, respectively.

RESULTS

Here we performed antibody interference experiments to investigate the role of Survivin in regulating the assembly of metaphase spindles. Partially purified polyclonal antibodies raised against full-length *Xenopus* Survivin were added at various concentrations to *Xenopus* egg extracts prior to initiating spindle

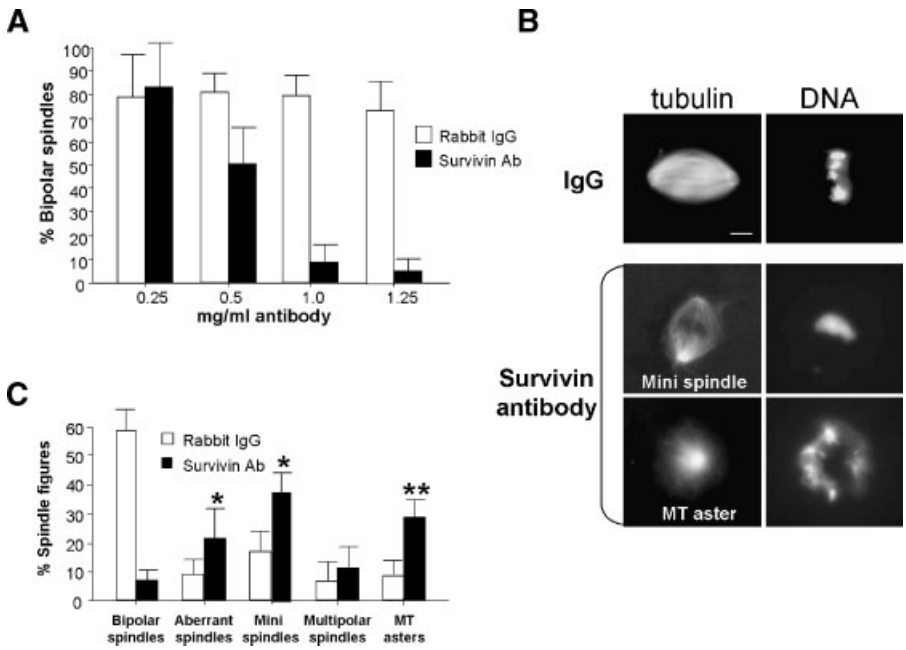

Fig. 1. Survivin antibody interferes with spindle formation in *Xenopus* egg extracts. **A**: Spindle assembly is specifically inhibited with increasing concentrations of Survivin antibodies but not rabbit IgG. Similarly, the specificity of spindle inhibition by the Survivin antibody was confirmed by pre-incubating the antibody with GST-Survivin fusion protein (see Materials and Methods). Results are expressed as the mean ± standard deviation (SD) for four independent experiments. Spindle structures (75–100) were counted for each experiment. **B**: Representative examples of abnormal spindle phenotypes generated by treatment with Survivin antibodies (1.25 mg/ml final). Scale bar, 10 μm. **C**: Quantification of spindle abnormalities induced by anti-Survivin antibody interference. Values are the mean ± SD for four independent experiments with 75–100 spindles counted for each experiment. *, Mean for mini spindle phenotype was significantly different ($P < 0.05$) by the student's *t*-test. **, Mean for MT asters was statistically highly significant ($P < 0.01$).

assembly reactions. As a negative control, similar concentrations of rabbit IgG purified from pre-immune serum were added to egg extracts in parallel. The efficiency of spindle assembly in non-treated egg extracts was typically in the range of 70%–85% depending on the quality of the egg extract.

Figure 1A shows that, treatment of *Xenopus* egg extracts with anti-Survivin antibodies, interfered with spindle assembly in a concentration-dependent manner. At antibody concentrations of 1–1.25 mg/ml; spindle assembly was strongly reduced to 5%. In contrast, spindle assembly proceeded efficiently in egg extracts treated with equivalent concentrations of pre-immune antibodies indicating that the inhibition of spindle formation is probably due to the presence of Survivin antibodies.

Next, we characterized the abnormal spindle structures in egg extracts treated with anti-Survivin or rabbit IgG antibodies. The spindle abnormalities fell into four categories: aberrant spindles (curved or narrow in appearance), shortened 'mini' spindles, multipolar spindle structures, and MT asters (see Fig. 1B,C). Of the four spindle phenotypes, there was an increase in aberrant spindles (21% vs. 9%), shorten spindles (38% vs. 17%), and MT asters (~30% vs. 9%) for extracts treated with Survivin antibodies compared to extracts treated with rabbit IgG (Fig. 1B). Thus, the addition of anti-*Xenopus* Survivin antibodies to egg extracts interferes with normal spindle assembly leading to aberrant spindles, shorten spindle structures, and MT asters.

As a second approach, Survivin antibodies were used to deplete most of the endogenous Survivin protein from *Xenopus* egg extracts. Briefly, CSF-arrested egg extracts were incubated with anti-Survivin antibodies pre-bound to protein A agarose beads. Typically, two rounds of immunodepletion were required to remove at least 90% of the endogenous Survivin (Fig. 2A). Spindle assembly reactions were then performed in the twice-depleted CSF-arrested egg extracts. Despite the reduced efficiency for spindle assembly in general (probably due to excessive manipulations of the extracts), our results show that depletion of Survivin greatly suppressed spindle assembly compared

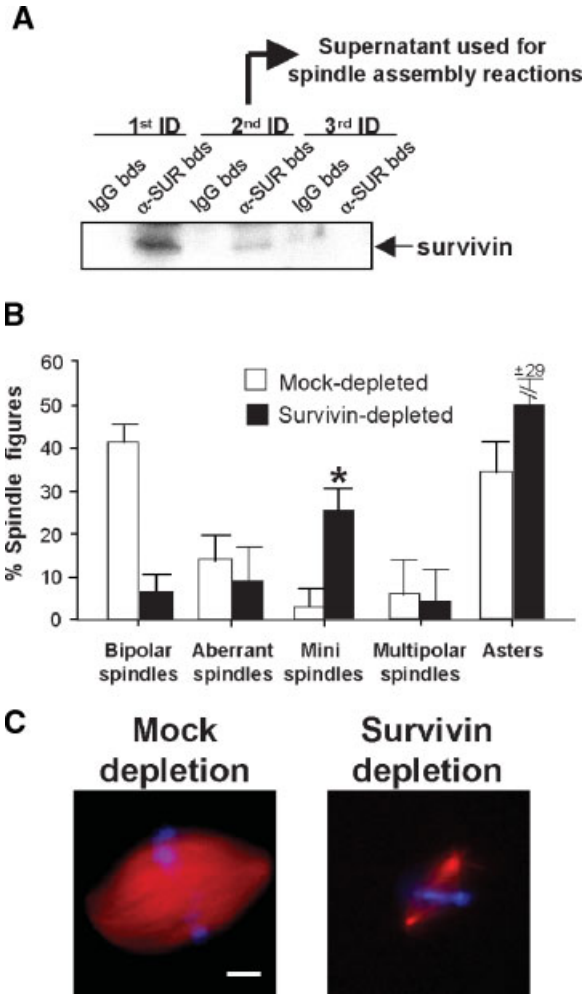

Fig. 2. Survivin is required for spindle assembly. *Xenopus* CSF-arrested egg extracts were immunodepleted (ID) of endogenous Survivin with two rounds of anti-Survivin antibodies bound to protein A beads. To estimate the amount of Survivin remaining after two rounds of depletion, a portion of the extract was subjected to a third round of depletion and the antibody bead complexes were immunoblotted with anti-Survivin antibodies. Generally, 5% or less of Survivin was remaining after two rounds of depletion. The remaining twice-depleted extracts (Survivin- or IgG-depleted) were used for spindle assembly reactions. Sperm DNA (200/μl final) and rhodamine-labeled tubulin were added to the depleted extracts and incubated for 75–90 min at room temperature (23°C) to allow metaphase spindles to form. After 75–90 min, spindle structures were fixed with formaldehyde onto microscope slides and visualized by fluorescence microscopy. **A**: Survivin bound to protein A agarose beads after each round of depletion (ID1, ID2, and ID3) was assessed by immunoblot analysis. **B**: Quantification of abnormal phenotypes of spindles formed in Survivin-depleted extracts. Data represents the mean ± SD of three independent experiments in which 100 or more spindles were counted for each experiment. *, Mean of mini spindle phenotype is statistically significant ($P < 0.05$) by the student's *t*-test. Results are representative of at least three independent experiments. **C**: Photos of control or mini spindle structures. Scale bar, 10 μm.

to mock-depleted extracts (5% vs. 38.5%; Fig. 2B). Several attempts to rescue spindle assembly by adding back recombinant *Xenopus* Survivin protein were unsuccessful. We attribute this to the twice-depleted extracts being too fragile for spindle assembly and to the probable removal of limiting factor(s) bound tightly to Survivin (see Discussion). Nevertheless, reducing the levels of Survivin from *Xenopus* egg extracts lead to a significant increase (~eightfold) of abnormal 'mini' spindle structures compared to IgG mock-depleted controls (Fig. 2B). Thus, our results indicate that Survivin is critical for regulating proper spindle assembly.

Closer inspection of the abnormal mini spindle structures that formed in the Survivin-depleted extracts revealed two striking features. First, the pole-to-pole length was on average significantly shorter for spindles formed in Survivin-depleted egg extracts when compared to spindles of control egg extracts. Specifically, the mean length of spindle structures formed in Survivin-depleted egg extracts was approximately 30 μM compared to 40 μM (pole-to-pole) for spindles assembled in control egg extracts. Moreover, 50% of the abnormal spindle structures that formed in Survivin-depleted extracts measured 30 μM or less in length compared to ~6% for control spindles (Fig. 3A). A second feature of the shortened spindles was the reduction of MTs around the mitotic chromatin (Fig. 3B). This was determined by measuring the fluorescence intensity of rhodamine-labeled tubulin from pole-to-pole as an indication of the MT density across the spindle.

Line scan plots of three independent mini spindles show an average fourfold decrease in fluorescence intensity at the mid section of the spindle structures indicating a lower MT density (Fig. 3C). This decrease in tubulin fluorescence was not dependent on the length of the spindle since longer spindle structures that formed in Survivin-depleted egg extracts also displayed a similar decrease in MTs around the mitotic chromatin (data not shown). In comparison, the MT density of many control spindles was found to be relatively constant from pole-to-pole (Fig. 3B,C).

Presumably the lower density of MTs in the center of the spindles is due to poor MT-chromatin association in Survivin-depleted egg extracts. To test this possibility, equivalent

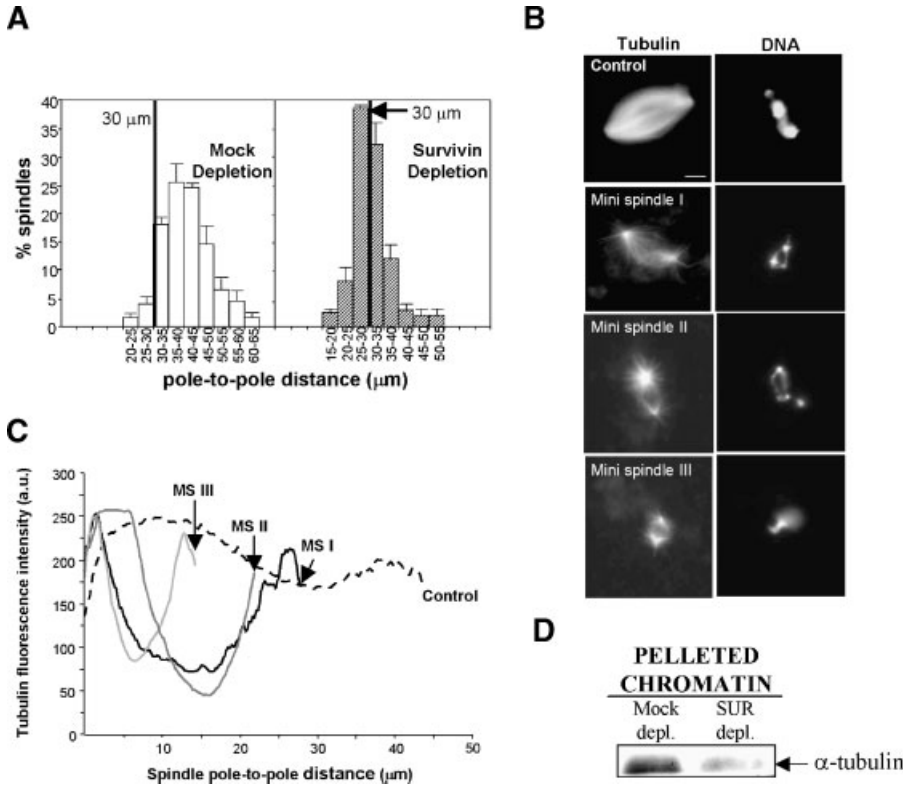

Fig. 3. Quantitative and qualitative analysis of mini spindles. **A**: Spindle size distribution in Survivin-depleted versus mock-depleted extracts. Results represent the mean ± SD of two independent experiments in which at least 200 spindles were counted for each experiment. **B**: Representative control (mock-depleted) spindle and three mini spindles (Survivin-depleted extracts) used to measure fluorescence intensity of microtubules (MTs) for part **C**. Scale bar, 10 μm. C: Plot showing fluorescence intensity of MTs from pole-to-pole of individual spindle structures shown in part B MS (mini spindle). Fluorescence intensity of tubulin is expressed in arbitrary units (a.u.). **D**: Tubulin associated with chromatin isolated from either mock- or Survivin-depleted egg extracts. Equal protein loading was confirmed by coomassie staining of membrane (data not shown).

volumes of Survivin-depleted or mock-depleted M phase egg extracts were collected following spindle assembly reactions. Chromatin was pelleted through a glycerol cushion and associated MTs were measured by immunoblot analysis of tubulin. As shown in Figure 3D, there is a substantial decrease in tubulin associated with chromatin from Survivin-depleted extracts. Thus, our data indicates that Survivin promotes the stabilization or binding of MTs to mitotic chromatin.

A critical requisite for Survivin function is the phosphorylation on its Thr34 residue by Cdc2/cyclin B [O'Connor et al., 2000]. The Cdc2 phosphorylation site is conserved in Survivin of all vertebrates and in *Xenopus* Survivin is found at residue Thr43 (Supplementary Fig. S1). Using site-directed mutagenesis, we engineered a non-phosphorylatable Survivin mutant by changing the Thr43 residue to alanine (SURT43A mutant). Alternatively, a phospho-mimicking mutation was introduced by substituting a glutamate residue in place of Thr43 (SURT43E mutant). Finally, we created a COOH-terminal truncation of Survivin by deleting the last 51 amino acids containing the α-helical coiled-coil domain. We refer to this coil-less Survivin mutant as SURCL. A schematic representation of all Survivin mutants is illustrated in Figure 4A.

Each of the Survivin mutants were expressed in *E. coli* as recombinant GST-Survivin$(His)_6$ fusion proteins and purified by sequential nickel chelate and glutathione column chromatography. To determine whether phosphorylation of the Thr43 residue in *Xenopus* Survivin played a role in regulating the formation of metaphase spindles, we added ~tenfold excess Gst-SURT43A$(His)_6$ (300 nM final) to *Xenopus* egg extracts prior to initiating spindle assembly reactions. The results show that spindle assembly was inhibited by at least 60% (Fig. 4B). The addition of the Gst-SURT43A$(His)_6$ mutant to egg extracts promoted the formation of a

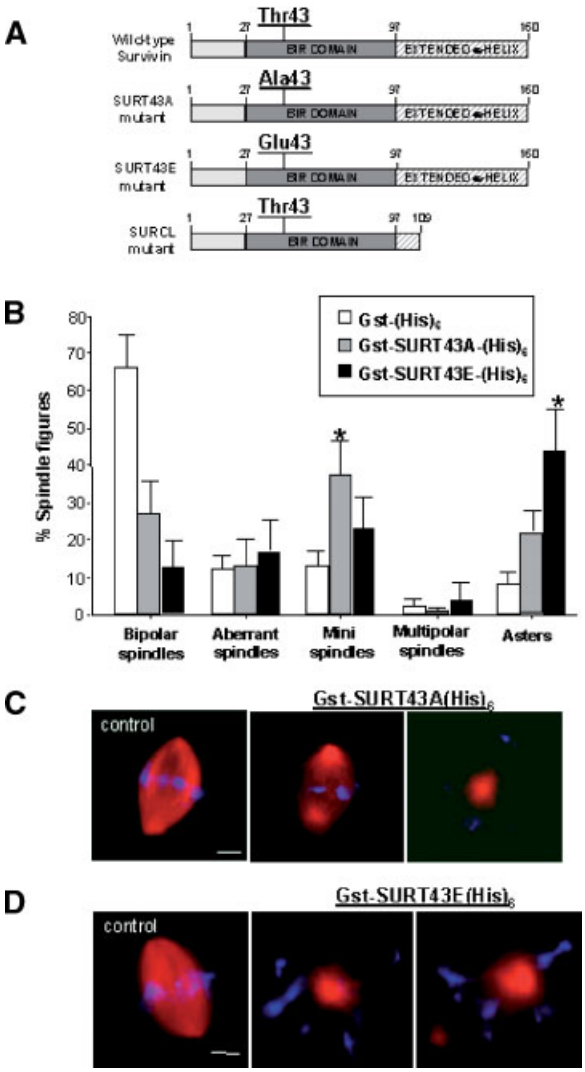

Fig. 4. The conserved Cdc2 phosphorylation site of Survivin is critical for regulating spindle assembly. **A**: Schematic diagram of Survivin mutant constructs. **B**: Quantification of spindle abnormalities induced by SURT43A and SURT43E mutants. Non-phosphorylatable [Gst-SURT43A(His)$_6$] or phospho-mimicking [Gst-SURT43E(His)$_6$] Survivin mutants were added to *Xenopus* egg extracts at a final concentration of 300 nM prior to spindle assembly reactions. Data represent the mean ± SD of at least three experiments. *, Mean shows a significant difference ($P < 0.05$) to controls. For each experiment 100–150 spindle figures were counted per sample. **C**: Representative mini spindle and MT aster that form in Gst-SURT43A(His)$_6$-treated extracts. Scale bar, 10 μm. **D**: Typical MT aster or MT bundle that form in Gst-SURT43E (His)$_6$-treated extracts. Scale bar, 10 μm.

significant number of mini spindle structures, and, to a lesser extent, MT asters. compared to control extracts treated with GST-(His)$_6$ tag alone (Fig. 4B). The addition of wild-type GST-Survivin(His)$_6$ protein to egg extracts had a minimal effect on spindle assembly (see Fig. 5B) indicating that the mini spindles formed by the Gst-SURT43A(His)$_6$ mutant were probably due to the absence of the Thr43 phosphorylation site.

Next, we tested whether the addition of a phospho-mimicking Survivin mutant (SURT43E) had any effect on spindle assembly. To our surprise spindle assembly was strongly inhibited in SURT43E-treated extracts (reduced to 12% efficiency). A substantial number of MT asters or structures resembling MT bundles were generated in SURT43E-treated egg extracts compared to control treated extracts (fivefold increase; Fig. 4B,D). Thus, mutations that mimic or block phosphorylation of the Thr-43 residue of *Xenopus* Survivin can disrupt spindle assembly. We conclude that the conserved Thr-43 phosphorylation site in *Xenopus* Survivin is important for proper spindle assembly.

Lastly, we examined the effect of the Survivin coil-less mutant (SURCL) on spindle assembly. As predicted from the absence of its tubulin-binding domain [Li et al., 1998], the SURCL mutant does not associate with the MT fraction when incubated in *Xenopus* egg extracts (Fig. 5A, top panel). In contrast, full-length wild-type and phosphorylation Survivin mutants are readily detected in the pelleted MT fraction. The addition of the SURCL to *Xenopus* egg extracts blocked spindle assembly by greater than 50%. Furthermore, statistical analysis showed a highly significant difference ($P < 0.01$) in the frequency of mini spindle structures generated in SURCL-treated egg extracts relative to control extracts treated with either wild-type Gst-SURwt(His)$_6$ or Gst-(His)$_6$ tag proteins (Fig. 5B,C). The mini spindle phenotype is very similar to that observed in earlier experiments that remove or inhibit Survivin function in egg extracts indicating that the effect of the SURCL mutant is through disrupting endogenous Survivin activity. Thus, our results suggest that the SURCL coil-less mutant acts as a dominant negative to disrupt normal spindle assembly.

Survivin localizes to the inner centromere regions of mitotic chromosomes during the early stages of mitosis [Uren et al., 2000; Morishita et al., 2001; Temme et al., 2003]. In *Xenopus* egg extracts both Survivin and Aurora B activity (as measured by phospho-H3) became enriched at the chromatin during mitosis (Fig. 5D). Consistent with this observation recombinant wild-type Survivin also associated with the chromatin fraction (Fig. 5E). In contrast, the SURCL

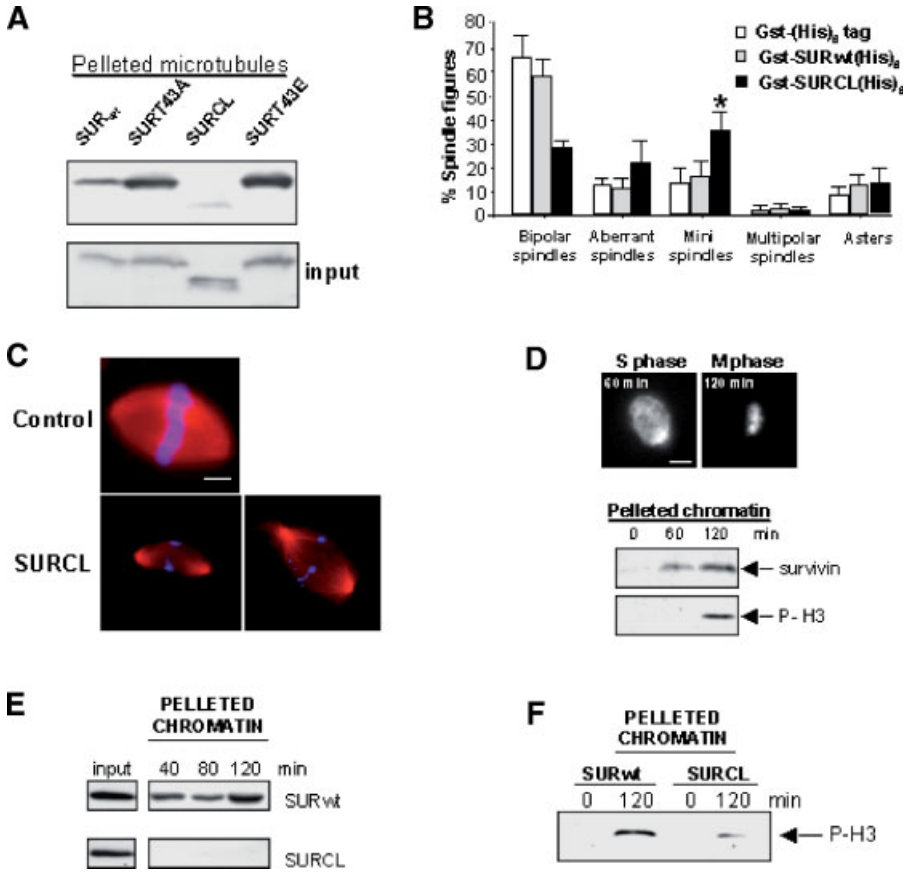

Fig. 5. Analysis of the coil-less Survivin mutant in *Xenopus* egg extracts. **A**: Coil-less Survivin mutant fails to bind MTs. Equal amounts of recombinant wild-type or indicated GST-Survivin(-$His)_6$ mutant proteins were incubated in *Xenopus* mitotic extracts for 30 min. The MT fraction was isolated as described [Horne and Guadagno, 2003] and recombinant proteins were detected by immunoblot analysis with anti-His tag antibodies (**top panel**). **Bottom panel** represents input of recombinant Survivin proteins levels in extracts prior to pelleting MTs. **B**: Coil-less Survivin mutant promotes mini spindles in *Xenopus* egg extracts. Quantitative analysis of spindle structures formed in *Xenopus* egg extracts treated with 300 nM recombinant Gst-SURCL$(His)_6$, Gst-SURwt$(His)_6$, or Gst-$(His)_6$ alone. Results represent the mean $\pm$ SD of at least four independent experiments. *, Statistically highly significant difference ($P < 0.01$). Over 100 spindle structures counted per experiment. **C**: Mini spindle structures that form in Gst-SURCL$(His)_6$-treated extracts. Control shown represents Gst-$(His)_6$-treated extracts. Scale bar, 10 μM. **D**: Survivin localizes to the chromatin at mitosis and correlates with the presence of phosphorylated histone H3, a marker of Aurora B activity and mitosis. Cycling of CSF extracts was initiated by the addition of 0.4 mM $CaCl_2$. At indicated times chromatin was isolated from 25-μl aliquots of crude egg extract and analyzed for bound Survivin and p-H3 using anti-Survivin and α-phospho-H3 antibodies. Cell-cycle state was monitored by nuclear envelope breakdown and chromatin condensation (top panel). Scale bar, 10 μm. **E**: The coil-less mutant fails to associate with chromatin in *Xenopus* egg extracts. Recombinant Gst-SURwt$(His)_6$ or Gst-SURCL$(His)_6$ proteins were incubated with cycling CSF egg extracts. At indicated times, chromatin was isolated from 25-μl aliquots of extract, solubilized in SDS sample buffer, and equal amounts of protein (as assessed by coomassie staining) were analyzed for recombinant Survivin by immunoblot analysis or subjected to immunoblot analysis for phospho-H3 levels (**F**).

coil-less mutant was undetected in the chromatin fraction following its incubation in *Xenopus* egg extracts (Fig. 5E, bottom panel). As Survivin is important for the localization and activation of Aurora B to the inner centromere regions of mitotic chromosomes [Morishita et al., 2001; Honda et al., 2003; Lens et al., 2003], we asked whether the presence of excess SURCL mutant protein in *Xenopus* egg extracts interfered with Aurora B activity at the chromatin. Equivalent amounts of wild-type (SURwt) or SURCL mutant protein (300 nM) were added to *Xenopus* egg extracts prior to cycling into mitosis. Chromatin from equal volumes of egg extract was isolated and subjected to Western analysis with phospho-H3 antibodies. Equivalent loading of chromatin-associated proteins for each sample was determined by coomassie blue staining (data not shown). The results (Fig. 5F) show that phospho-H3 was markedly

reduced in chromatin isolated from extracts containing excess SURCL mutant indicating a reduction in chromatin-associated Aurora B activity. Thus, our data suggest that the addition of the SURCL mutant to egg extracts prevents the accumulation of Aurora B activity at the chromatin.

DISCUSSION

Cellular studies have provided only superficial evidence linking Survivin to regulation of the mitotic spindle. In this study we exploited the powerful cell-free system of *Xenopus* egg extracts to ask whether Survivin is directly required for spindle assembly. Several independent approaches were used to inhibit Survivin in *Xenopus* egg extracts: antibody interference, immunodepletion, and addition of dominant-negative acting Survivin mutants. Regardless of the approach, spindle assembly was inhibited 55%–95% resulting in the formation of mini spindle structures and, to a lesser extent, MT asters. In addition, we show that regulation of Survivin phosphorylation at the Thr43 residue and its tubulin-binding domain play crucial roles in mediating the spindle assembly functions of Survivin. Taken together, our work further validates Survivin's involvement in spindle regulation and directly shows for the first time its requirement for regulating spindle assembly per se.

Regulation of Survivin Phosphorylation Contributes to Spindle Regulation

Phosphorylation on Thr34 of human Survivin by the mitotic kinase Cdc2/cyclin B is an important requirement for its in vivo functions [O'Connor et al., 2000]. Mutation of the Thr34 site to an alanine residue and its overexpression in cells prevents phosphorylation of endogenous Survivin, causes defective cytokinesis, and promotes the induction of tumor cell apoptosis suggesting that it acts as a dominant negative [Li et al., 1998; O'Connor et al., 2000; Skoufias et al., 2000; Mesri et al., 2001; Fortugno et al., 2002; Temme et al., 2003]. Similarly, *Xenopus* Survivin contains an equivalent phosphorylation site at residue Thr43 (see supplementary Fig. S1) and is able to associate with Cdc2/cyclin B at mitosis in cycling egg extracts (supplementary Fig. S3). Mutation of the Thr43 phosphorylation site to an alanine residue had an appreciable inhibitory effect (60% decrease) on spindle assembly in *Xenopus* egg extracts (Fig. 4). The main spindle abnormality produced in the presence of the SURT43A mutant resembles the mini spindle structures that also formed in Survivin-depleted egg extracts (compare Figs. 2 and 3 vs. Fig. 4C) suggesting that the SURT43A mutant acts in a dominant-negative manner.

To our surprise, the phospho-mimicking mutation (SURT43E) also had a potent effect on spindle assembly in *Xenopus* egg extracts. As the SURT43E mutant mimics a constitutively phosphorylated 'active' form of Survivin, we propose that it interferes with spindle assembly at an early stage. Consistent with this proposal, a much higher frequency of MT asters form with the SURT43E mutant compared to the phosphorylation-defective mutant (see Fig. 4B,C). Thus, our results indicate that the timing of Survivin Thr43 phosphorylation during mitosis is important in regulating spindle assembly.

The C-terminal Tubulin-Binding Domain of Survivin Is Required for Spindle Assembly

Survivin localizes to various components of the spindle apparatus including the centrosomes, spindle pole MTs, and kinetochores at metaphase, the spindle midzone at anaphase, and the midbody during cytokinesis [Li et al., 1998; Skoufias et al., 2000; Fortugno et al., 2002]. Its extended COOH terminus α-helical coiled-coil domain is important for dimerization [Muchmore et al., 2000] and binding to polymerized tubulin [Li et al., 1998]. We deleted the α-helical coiled-coil domain in *Xenopus* Survivin and, as predicted, showed that the deletion mutant (SURCL) poorly associates with the MT fraction isolated from *Xenopus* M phase egg extracts compared to full-length Survivin (Fig. 5A). The addition of the SURCL mutant to egg extracts disrupted spindle assembly giving rise to mini spindle structures that are similar in appearance to those observed in Survivin-depleted egg extracts (Figs. 2B and 5C). These results indicate that the tubulin-binding domain of Survivin serves a role in spindle assembly by properly localizing Survivin to the spindle MTs or by possibly interfering with the dimerization of endogenous Survivin.

Proposed Roles for Survivin in Spindle Assembly

Spindle assembly in *Xenopus* egg extracts starts with the formation of MT asters that are generated from sperm-associated centrosomes.

Following aster formation, the MTs attach to chromosomes and become stabilized to allow for the maturation of a bipolar spindle (see Fig. 6). Evidence from our study suggests that Survivin activity is important at these steps in spindle assembly. Depleting or inhibiting Survivin in *Xenopus* egg extracts gives rise to either MT asters or mini spindle structures as the two predominant phenotypes. A striking feature of the mini spindles is the reduction of MT density around the mitotic chromatin. Further, we showed that the amount of tubulin associated with chromatin isolated from Survivin-depleted egg extracts was dramatically reduced compared to control egg extracts (Fig. 3D). While these spindle defects could be due to secondary effects of the Survivin depletion, it should be noted that similar abnormalities in spindle assembly were observed by the addition of Survivin antibody or excess Survivin T43A and C-terminal deletion mutants (see Figs. 1B, 4C, and 5C). Taken together, we propose that Survivin may act to stabilize MT–chromatin interactions during the formation of the bipolar spindle. Studies in HeLa cells have reported similar spindle alterations as a consequence of targeting Survivin by siRNA [Honda et al., 2003] or micro-injected with Survivin antibodies [Giodini et al., 2002]. Thus, we conclude that maturation of the bipolar spindle requires Survivin.

How might Survivin promote the stabilization of MTs around the mitotic chromatin? Survivin is a component of the chromosome passenger complex (Survivin/inner centromere protein (INCENP)/Aurora B) [Adams et al., 2000; Kaitna et al., 2000; Wheatley et al., 2001; Bolton et al., 2002] that binds to the centromere domain of chromosomes during mitosis. The chromosomal passenger complex is implicated in chromosome alignment, spindle checkpoint controls, chromatin-induced MT stabilization, and spindle assembly [Carmena and Earnshaw, 2003; Andrews et al., 2004; Gassmann et al., 2004; Sampath et al., 2004]. Though the requirement for Survivin in these previous studies was not addressed, other studies have shown that Survivin is required for the localization of Aurora B activity to the chromosomes [Honda et al., 2003; Lens et al., 2003]. We are able to show similar findings in *Xenopus* egg extracts with the dominant-negative SURCL mutant (Fig. 5F) or depletion of endogenous Survivin (data not shown). In addition, we predict that other downstream effectors of Survivin may be involved in spindle regulation. For example, Cdc2 plays a central role in regulating MT dynamics [Verde et al., 1990; Vasquez et al., 1999; Karsenti and Vernos, 2001]. As it has already been shown to interact and phosphorylate Survivin [O'Connor et al., 2000], Cdc2 recruitment to the spindle apparatus through its association and regulation of Survivin may also be an important regulatory step in spindle assembly. Thus, we propose that Survivin functions as an adaptor or scaffold-like protein that brings together and/or localizes spindle regulators to the mitotic spindle.

In summary, we conclude that Survivin activity is critical for mediating the proper assembly of the metaphase spindle. Based on the mini spindle morphology that arises as a consequence of inhibiting Survivin, we propose that Survivin promotes the stabilization of

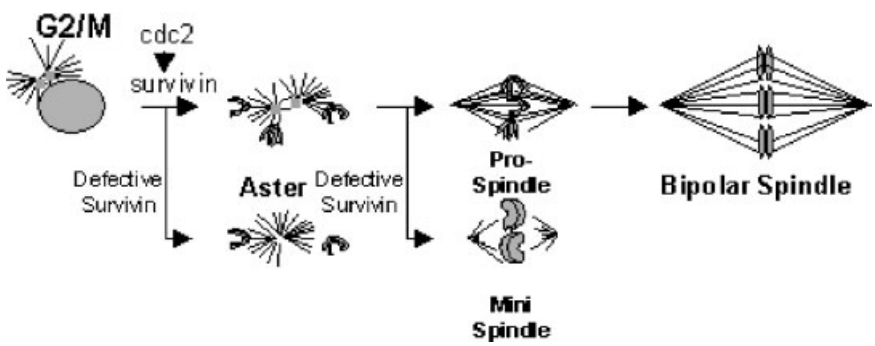

Fig. 6. Summary of proposed roles for Survivin in regulating spindle assembly. Survivin localizes at the centrosomes during late prophase. At prometaphase, Survivin relocalizes to the kinetochores where it functions to stabilize MT–chromosome interactions. The removal or interference of Survivin function leads to the formation of MT asters or mini spindle structures. Consequently, this would lead to a non-functional spindle and abnormal chromosome segregation.

MT–chromatin interactions. As Survivin is strongly deregulated in the vast majority of cancers through its overexpression, this may contribute to abnormal spindle attachments, aberrant chromosome segregation, and insensitivity to the spindle assembly checkpoint control.

ACKNOWLEDGMENTS

We thank Yanfen Chen for her excellent technical assistance in the preparation of recombinant proteins and the lab members for their helpful comments on the data. This work has been supported in part by the molecular imaging and molecular biology core facilities at the H. Lee Moffitt Cancer Center. A National Institute of Health grant (GM62542) to T.M.G. supported these studies.

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