Survivin is required for stable checkpoint activation in taxol-treated HeLa cells

Ana Carvalho[1,2,3], Mar Carmena[1], Clara Sambade[2,3], William C. Earnshaw[1,4,*] and Sally P. Wheatley[1]

[1]Chromosome Structure Group, Wellcome Trust Centre for Cell Biology, Institute of Cell and Molecular Biology, University of Edinburgh, King's Buildings, Mayfield Road, Edinburgh EH9 3JR, Scotland, UK
[2]University of Porto, IPATIMUP Institute of Molecular Pathology and Immunology of the University of Porto, Porto, Portugal
[3]Faculty of Medicine, University of Porto, Porto, Portugal
*Author for correspondence (e-mail: bill.earnshaw@ed.ac.uk)

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Summary

Survivin is an essential chromosomal passenger protein whose function remains unclear. Here, we have used RNA interference to specifically repress Survivin in cultured HeLa cells. Immunoblot analysis showed that Survivin was no longer detectable in cultures 60 hours after transfection with Survivin-specific siRNA. Live cell analysis showed that many Survivin-depleted cells were delayed in mitosis, and immunofluorescence analysis of fixed specimens revealed that Survivin-depleted cells accumulated in prometaphase with misaligned chromosomes. The chromosomal passenger proteins, INCENP and Aurora-B, which can interact directly with Survivin, were absent from the centromeres of Survivin-depleted cells. These data contribute to the emerging picture that Survivin operates together with INCENP and Aurora-B to perform its mitotic duties. Some Survivin-depleted cells eventually exited mitosis without completing cytokinesis. This resulted in a gradual increase in the percentage of multinucleated cells in the culture. Time-lapse imaging of synchronized cultures revealed that control and Survivin-depleted cells arrested in mitosis in the presence of nocodazole; however, the latter failed to arrest in mitosis when treated with taxol. Immunofluorescence studies revealed that Survivin-depleted cells were unable to stably maintain BubR1 at the kinetochores in the presence of either taxol or nocodazole. Our data reveal that Survivin is not required for the spindle assembly checkpoint when it is activated by the loss of microtubules. However, Survivin is required for the maintenance of the checkpoint when it is activated by taxol, which is generally thought to cause a loss of spindle tension.

Key words: Mitosis, Chromosomal passenger proteins, Survivin, Aurora-B, INCENP, RNA interference, BubR1

Introduction

Since its discovery by Altieri and co-workers (Ambrosini et al., 1997), considerable effort has been directed at understanding the function of Survivin. This small protein (16.5 kDa) is only expressed in embryonic or proliferating adult tissues and is highly overexpressed in many forms of cancer (Altieri, 2001). Although Survivin has a prominent BIR domain, consistent with a potential role in apoptotic regulation, the principle defects manifested upon interference with Survivin function are in mitosis (for reviews, see Altieri et al., 1999; Silke and Vaux, 2001; Reed and Bischoff, 2000).

Recently Survivin has been shown to be a chromosomal passenger protein (Skoufias et al., 2000; Uren et al., 2000; Jiang et al., 2001; Wheatley et al., 2001a). These highly conserved proteins are present at centromeres during prometaphase, then transfer to the central spindle and presumptive cleavage furrow at the cell cortex during early anaphase (see Earnshaw and Bernat, 1990; Earnshaw and Cooke, 1991; Adams et al., 2001a). Chromosomal passenger proteins cloned to date include INCENP/Sli-15, Aurora-B/Ipl-1/AIR-2 and Survivin/BIR1 (for reviews, see Adams et al., 2001a; Terada, 2001), but at least one other protein with similar behaviour also exists, TD60 (telophase-disc 60) (Andreassen et al., 1991).

INCENP is a multidomain protein that interacts with heterochromatin protein-1 (Ainsztein et al., 1998) and microtubules (Wheatley et al., 2001b), and it is thought to be a targeting or scaffolding subunit for Aurora-B kinase (Adams et al., 2000; Bolton et al., 2002). In contrast, relatively little is known about the mitotic role of Survivin other than that it can stimulate Aurora-B kinase activity in vitro (Bolton et al., 2002; Bishop and Schumacher, 2002). It is likely that the chromosomal passengers act in concert to execute their role(s) during mitosis, and they are stockpiled as a complex in *Xenopus* eggs and in yeast (Adams et al., 2000; Bolton et al., 2002; Cheeseman et al., 2002). Interactions between the three proteins have also been detected by two-hybrid and in vitro binding experiments (Kaitna et al., 2000; Wheatley et al., 2001a).

INCENP and Aurora-B are required for phosphorylation of histone H3 on serine 10 (Hsu et al., 2000; Adams et al., 2001b; Murnion et al., 2001) and, in *Drosophila* and *S. pombe* [though not *Xenopus* (MacCallum et al., 2002)], for targeting of condensin to chromosomes (Giet and Glover, 2001; Morishita et al., 2001). The passengers are essential for chromosomes to achieve a stable metaphase biorientation (Adams et al., 2001b; Kaitna et al., 2002), possibly by regulating the interaction of kinetochores with microtubules (Yoon and Carbon, 1999;

Biggins et al., 1999; Biggins and Murray, 2001; Kang et al., 2001; Tanaka et al., 2002; Kaitna et al., 2002). Ipl-1p (the budding yeast Aurora kinase) is essential for the tension-sensitive arm of the spindle assembly checkpoint (Biggins and Murray, 2001), and studies using dominant-negative mutants and antibody injection have implicated mammalian Survivin and Aurora-B in the spindle assembly checkpoint (Murata-Hori et al., 2002; Kallio et al., 2001; Kallio et al., 2002). Aurora-B has been implicated in anaphase spindle midzone organization in many species (Speliotes et al., 2000; Giet and Glover, 2001; Murata-Hori et al., 2002; Murata-Hori and Wang, 2002; Giodini et al., 2002) and spindle elongation in yeast (Rajagopalan and Balasubramanian, 2002). Survivin may also contribute to the regulation of microtubule dynamics (Li et al., 1998; Giodini et al., 2002) and has been reported to be involved in spindle assembly (Giodini et al., 2002). In *C. elegans*, Aurora-B/AIR-2 is also required for separation of homologous chromosomes in meiosis I (Rogers et al., 2002; Kaitna et al., 2002). Lastly, the passengers are required for the execution of cytokinesis (Mackay et al., 1998; Schumacher et al., 1998; Terada et al., 1998; Cutts et al., 1999; Fraser et al., 1999; Li et al., 1999; Speliotes et al., 2000; Uren et al., 2000; Oegema et al., 2001; Leverson et al., 2002).

In the present study we have used siRNA (Elbashir et al., 2001) to silence Survivin expression in human cells. Our results indicate that Survivin is required for metaphase chromosome alignment and maintenance of the spindle assembly checkpoint arrest in the presence of taxol and cytokinesis. Furthermore, we show that a monoclonal antibody (Fortugno et al., 2002) used in many studies of Survivin localization apparently recognizes another spindle-associated epitope(s) in addition to Survivin.

Materials and Methods

Oligonucleotides

A 21-mer oligonucleotide (ggaccaccgcaucucuacadtdt) covering bases 45-65 downstream of the translational start codon of human Survivin cDNA (Acc. No. U75285) was selected as the targeting sequence according to the criteria described previously (Elbashir et al., 2001). This sequence is unique to human Survivin and is present in the mRNA from all known Survivin splice variants (Mahotka et al., 1999). A 21-mer oligonucleotide (cguacgcggaauacuucgadtdt) labelled with or without rhodamine that had no significant homology to any known human mRNA in the databases was used as control (Elbashir et al., 2001). Double-stranded RNA (dsRNA) molecules were supplied as annealed duplexes (www.xeragon.com).

Cell culture, RNAi application and drug treatments

HeLa cells in exponential growth were seeded onto poly-lysine-coated glass coverslips in 24-well plates at 3×10^4 cells per well and grown overnight in RPMI/10% FBS (Gibco-BRL) and maintained in 5% CO_2 at 37°C. HeLa cells stably expressing Survivin-GFP were described previously (Wheatley et al., 2001a). RNAi was performed according to Elbashir et al., (Elbashir et al., 2001) (see also www.mpibpc.gwdg.de/abteilungen/100/105/). Briefly, a single pulse of 60 pmoles of siRNA was administered to the cells at 50% confluency by transfection with OligoFectamine (www.invitrogen.com) in complete medium without antibiotics. Cells were maintained in this medium for the duration of the experiment and assayed for Survivin silencing by indirect immunofluorescence and immunoblotting. For synchronisation in S-phase, cells were treated with 2 mM thymidine for 20 hours at 37°C then released into fresh medium. To depolymerize microtubules, cells were treated with either 0.1 μg/ml colcemid (Sigma) for 2-24 hours or 0.1 μg/ml nocodazole (Sigma) for 12-18 hours. To stabilise microtubules, cells were incubated with 33 nM taxol (Sigma) for 12-18 hours.

Immunoblotting

Total extracts of 5×10^5 cells treated with control or Survivin-specific oligonucleotides for 60 hours or 84 hours were boiled for 5 minutes in Laemmli buffer containing β-mercaptoethanol, run in a 15% SDS-PAGE gel and blotted onto a nitrocellulose membrane (Amersham; www.apbiotech.com). The membrane was blocked with 5% skimmed milk in PBS/0.1% Tween 20 and incubated with an anti-Survivin antibody (diluted 1/500; NB500-201 www.novus-biologicals.com) in 3% milk/PBS/0.1% Tween 20 for 3 hours at room temperature. After washing in 1.5% milk/PBS/0.1% Tween 20, the membrane was incubated for 1 hour with anti-rabbit horseradish-peroxidase-linked secondary antibody (diluted 1/10,000, www.apbiotech.com) in 3% milk/PBS/0.1% Tween 20. After washing, the membrane was labelled using the enhanced chemiluminescence protocol (www.apbiotech.com).

Indirect immunofluorescence microscopy and quantification

At various time points after transfection, cells were fixed with 4% formaldehyde and permeabilized with 0.15% Triton X-100 as described previously (Wheatley and Wang, 1996), unless otherwise stated. Cells were probed with antibodies against Survivin (1/500, rabbit; NB500-201 www.novus-biologicals.com), Aurora-B (1/250, mouse; AIM1 www.translab.com), INCENP (1/500; polyclonal antibody rabbit D), 8E2 (1/500, mouse, gift of D. Altieri, Yale University, USA), BubR1 (1/500, sheep; gift of S. S. Taylor, University of Manchester, UK), and α-tubulin (1/2000, mouse; B512 www.sigma-aldrich.com). Anti-centromeric antibodies (ACA) were used to stain the centromeres (1/1000, human), and chromosomes were stained with DAPI (Sigma). Mad2 staining was performed according to a previous protocol (Waters et al., 1998), using a polyclonal Mad2 antibody (1/50, rabbit; gift from E. D. Salmon, University of North Carolina, USA). Secondary antibodies were used at 1/200 (www.jacksonimmuno.com). Quadruple labelling was performed using a suitable combination of primary antibodies, with secondary antibodies conjugated to Texas Red, fluorescein and Cy5, and samples counterstained with DAPI. Chromosome spreads were made by hypotonically swelling colcemid-treated cells with 75 mM KCl for 20 minutes at room temperature prior to fixation.

Image stacks were taken using an Olympus IX-70 microscope controlled by Delta Vision SoftWorx (Applied Precision, Issequa, WA, USA) and a 60× objective (NA 1.4). Image stacks were deconvolved, quick-projected and captured as tiff images. For quantification, the 10 central sections of an image stack were deconvolved and projected using an averaging algorithm. The total integrated intensity of a 20×20 pixel box was measured at the appropriate wavelengths using the Data Inspector tool. For each prometaphase cell analysed, three measurements were taken on the chromosomes within the cell and three of the background outside the cell. Values were corrected by subtracting the background of the appropriate wavelength. Quantifications were made at the 48 hour or 60 hour time points so that Survivin-depleted cells could be compared directly with Survivin-positive cells on the same coverslip.

Time-lapse imaging

Cells were seeded onto poly-lysine-coated grid coverslips in 24-well plates at a density of 7.5×10^3 cells per well. Cells were blocked in S-phase with 2 mM thymidine for 20 hours, 40 hours after transfection. Cultures were washed several times to remove mitotic and apoptotic cells before adding medium with or without a drug (33 nM taxol or

0.1 μg/ml nocodazole) for 12-18 hours. When cells entered mitosis (~8-10 hours after release from the thymidine block), medium was supplemented with 10 mM HEPES (pH 7.5), and phase-contrast images were taken every hour using an inverted Nikon Diaphot microscope heated at 37°C and a 20× objective. At least 50 mitotic cells were followed for 6 hours in each experiment.

Cell population dynamics and cell cycle/apoptosis quantification

Growth curves were plotted based on the number of viable cells, as determined by Trypan blue exclusion (Sigma). Mitotic stages were scored separately for cells staining negatively [Survivin(–)] or positively [Survivin(+)] for Survivin. Both mitotic and apoptotic cells were scored based on DAPI morphology and α-tubulin immunostaining. As the precise timing of depletion varied from one experiment to another (typically 36-72 hours), representative graphs are shown to avoid blending of the data. All experiments were performed at least three times and produced similar results.

FACS analysis

The apoptotic status of cells was assessed using TUNEL label and Annexin V-FLUOS staining kits according to the manufacturer's guidelines (www.biochem.roche.com).

Before TUNEL labeling, the total population (including non-adherent cells) was washed in PBS, fixed in 4% formaldehyde for 30 minutes at room temperature and permeabilized in 0.1% sodium citrate/Triton X-100 for 2 minutes on ice.

To assess ploidy, cells were washed in PBS and fixed for 1 hour in 70% ethanol at 4°C. After treatment with 50 μg/ml RNAse (Sigma) for 20 minutes at room temperature, cells were washed with PBS and incubated with 40 μg/ml propidium iodide (Sigma) for 30 minutes.

All samples were analysed using a fluorescence activated cell sorter (FACSCalibur, Becton Dickinson, Mountain View, CA) and Cell Quest software.

Results

Successful repression of Survivin in HeLa cells using siRNA

To investigate the role of Survivin in mitosis, we have reduced the levels of this protein in exponentially growing adherent HeLa cells using RNA interference (RNAi). The target sequence was directed against nucleotides 45-65 of Survivin that code for a region common to the three known isoforms of the protein: Survivin, Survivin 2B and Survivin Delta Ex3 (Mahotka et al., 1999). This sequence was not detected in any other human gene by BLAST analysis. After transfection with Survivin-specific or control siRNA, cultures were harvested at 12 hours intervals from 24 hours, and the cell number was assessed (Fig. 1A). The growth rate of cells exposed to Survivin siRNA began to slow at 48 hours, and proliferation ceased after 60 hours. Similar data were obtained when colonies of cells were microinjected with the same oligonucleotides (data not shown). Immunoblot analysis using a polyclonal antibody previously shown to recognise all known forms of Survivin (Fortugno et al., 2002) revealed that Survivin was substantially repressed by 60 hours and 84 hours post transfection (Fig. 1B). We will refer to cells in which Survivin was not detected by indirect immunofluorescence as 'Survivin-depleted', but note that low levels of the protein may remain in such cells.

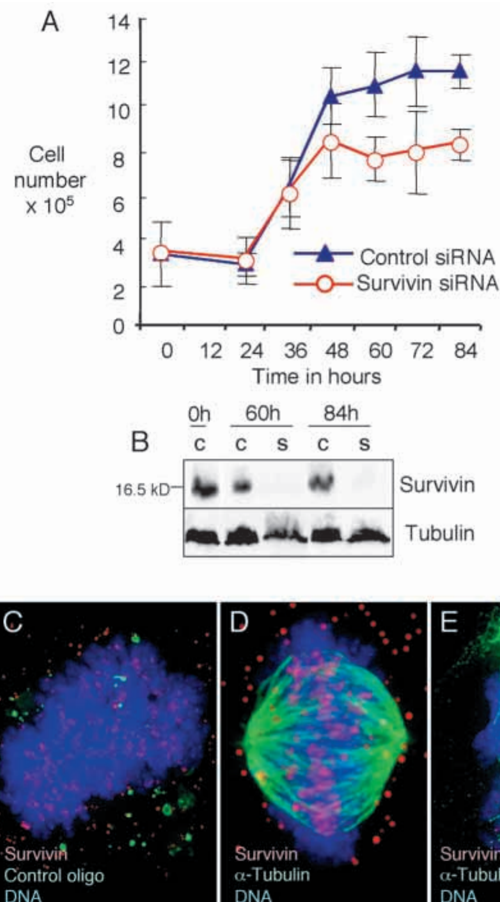

Fig. 1. Survivin can be depleted from HeLa cells using RNAi. (A) Growth curve of viable HeLa cells transfected with control siRNA (blue triangles) or Survivin siRNA (red circles). Cell number in Survivin RNAi population does not increase after 48 hours. (B) Immunoblot analysis of control (C) and Survivin-siRNA-transfected (S) cultures at 0 hours, 60 hours or 84 hours post transfection as indicated. Tubulin was monitored as a loading control. (C-E) Indirect immunofluorescence images showing Survivin in red, DNA in blue and tubulin in green. (C) Control cell transfected with rhodamine-labelled control siRNA (shown in green). Survivin (red) localises normally to the centromeres. (D) Survivin-positive cell from same culture as E, showing abundant Survivin at the centromeres. (E) Survivin-depleted cell from a Survivin siRNA-transfected population, fixed and stained at 60 hours. No Survivin (red) is detectable at the centromeres. Bars, 5 μm.

Following transfection with rhodamine-labelled control siRNA Survivin was detected at the centromeres of prometaphase/metaphase cells (Fig. 1C) and showed a typical chromosome passenger pattern of localization at other stages of mitosis (data not shown). In 50-75% (n=100) of prometaphase cells exposed to specific siRNA, Survivin was not detectable at centromeres by 48-60 hours post transfection (Fig. 1E). By contrast, untransfected cells from this population exhibited a normal Survivin distribution (Fig. 1D). We also transfected cells that stably express Survivin-GFP with control or Survivin siRNA. As shown in Fig. 2A, in cells transfected with control siRNA, Survivin-GFP was localized at the centromeres and was also diffuse in the cytoplasm as described previously (Wheatley et al., 2001a). By contrast, in cells

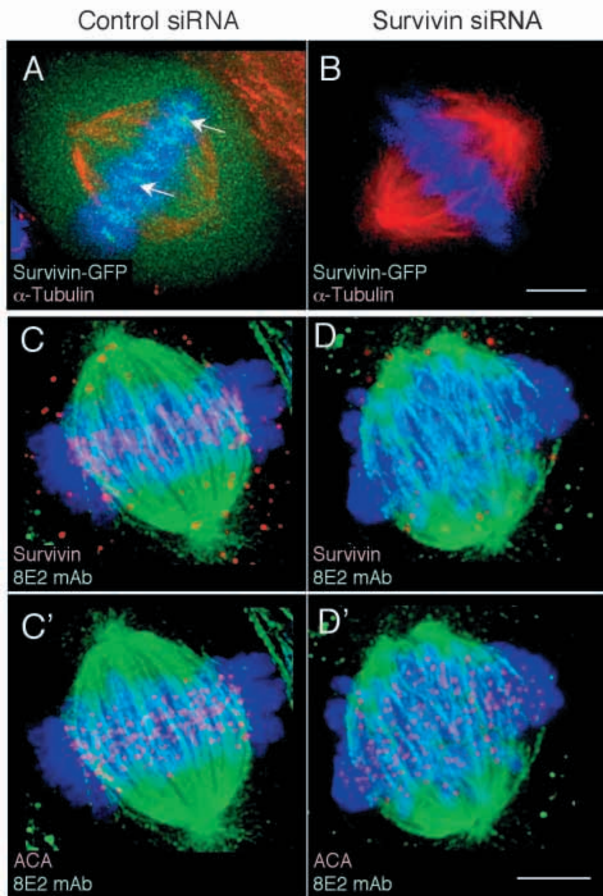

Fig. 2. Stably expressed Survivin-GFP, but not the epitope(s) recognized by the monoclonal antibody 8E2, can also be repressed by Survivin RNAi. (A,B) HeLa cells stably expressing Survivin-GFP (green) were subjected to Survivin RNAi and immunostained for tubulin (red) and counterstained with DAPI (DNA; blue). (A) Control cell with Survivin-GFP concentrated at the centromeres (arrows). (B) The Survivin-siRNA-transfected cell is depleted of Survivin-GFP signal. (C,C'-D,D') Control or Survivin-siRNA-transfected cells stained for Survivin using the polyclonal antibody (C,D in red) and centromeres (C',D' in red); the epitope(s) recognized by the monoclonal antibody 8E2 (green) and DNA (blue). Survivin-specific siRNA does not abolish spindle staining by 8E2. Bars, 5 μm.

exposed to Survivin siRNA, no GFP signal remained (Fig. 2B). This result was confirmed by immunoblot analysis using anti-GFP antibody (data not shown). We conclude that Survivin is essential for the growth of HeLa cells and that the protein can be depleted from cells by RNAi.

Staining of cells with a monoclonal antibody that detects microtubule associated Survivin is unaffected by Survivin-specific RNAi

An ongoing vigorous debate in the field is whether human Survivin is a microtubule associated protein, as originally described (Li et al., 1998; Li et al., 1999), a chromosome passenger protein, as subsequently claimed (Skoufias et al., 2000; Wheatley et al., 2001a; Uren et al., 2000) or whether two Survivin pools of different magnitude exist within the cell (Fortugno et al., 2002). An important reagent used in this debate has been the monoclonal antibody 8E2, which helped to define Survivin originally (Li et al., 1998; Li et al., 1999) and which recognizes a full-length Survivin fusion protein in vitro (Wheatley et al., 2001a).

A surprising result was obtained when we probed Survivin siRNA-treated cells with the 8E2 monoclonal antibody or with a commercial polyclonal antibody (NB500-201; www.novus-biologicals.com) reported to recognise all forms of Survivin (Fortugno et al., 2002). In cells transfected with control siRNA, the polyclonal antibody stained centromeric Survivin (Fig. 2C and ACA staining in Fig. 2C', red), whereas 8E2 demarcated the mitotic spindle (Fig. 2C,C', green). However, in cells negative for Survivin, as detected with the commercial polyclonal antibody (Fig. 2D, red), the pattern of 8E2 localization remained unaltered (Fig. 2D,D', green). Since we have shown that 8E2 can recognise Survivin in vitro (Wheatley et al., 2001a), this result strongly suggests that in vivo, 8E2 recognizes an epitope present on at least one other microtubule-associated protein in addition to survivin.

Survivin depletion causes mislocalisation of the chromosomal passenger proteins, Aurora-B and INCENP

It is now widely believed that INCENP and Aurora-B may act together with Survivin throughout mitosis in a chromosomal passenger complex (Wheatley et al., 2001a; Bolton et al., 2002; Leverson et al., 2002). As predicted by this, at 60 hours after transfection with specific siRNA, cells without detectable Survivin also lacked centromeric Aurora-B (Fig. 3B). A diffuse pool of Aurora-B and occasionally some residual staining of the chromosome arms was observed (compare Fig. 3A'' and Fig. 3B''). The availability of a mixed population of Survivin-positive and Survivin-depleted cells following transfection enabled us to use Survivin-positive cells as internal staining controls for quantitative analysis, which revealed a linear correlation between levels of Survivin and Aurora-B at the centromeres (Fig. 3E). A similar correlation was obtained between the levels of Aurora-B and INCENP at centromeres following transfection with Survivin siRNA (Fig. 3C,D,F). These data demonstrate that both Aurora-B and INCENP require Survivin to accumulate at centromeres during the early stages of mitosis.

Survivin depletion causes an accumulation of prometaphase cells with defects in chromosome congression

To determine the effect of depletion of Survivin on the passage of cells through mitosis, populations transfected with Survivin siRNA were stained for Survivin, and the percentage of cells in each phase of mitosis was scored. Survivin-positive cells (n=100 mitotics) maintained a wild-type distribution of mitotic stages for the duration of the experiment (Fig. 4A). In contrast, we observed a decline in the percentage of Survivin-depleted mitotic cells in metaphase, anaphase, telophase and cytokinesis from 36 hours post-transfection (Fig. 4B; n=100 mitotics). Instead, we observed many prometaphase cells with bipolar spindles but many maloriented chromosomes (Fig. 4D, arrows). Although cells with multipolar spindles were

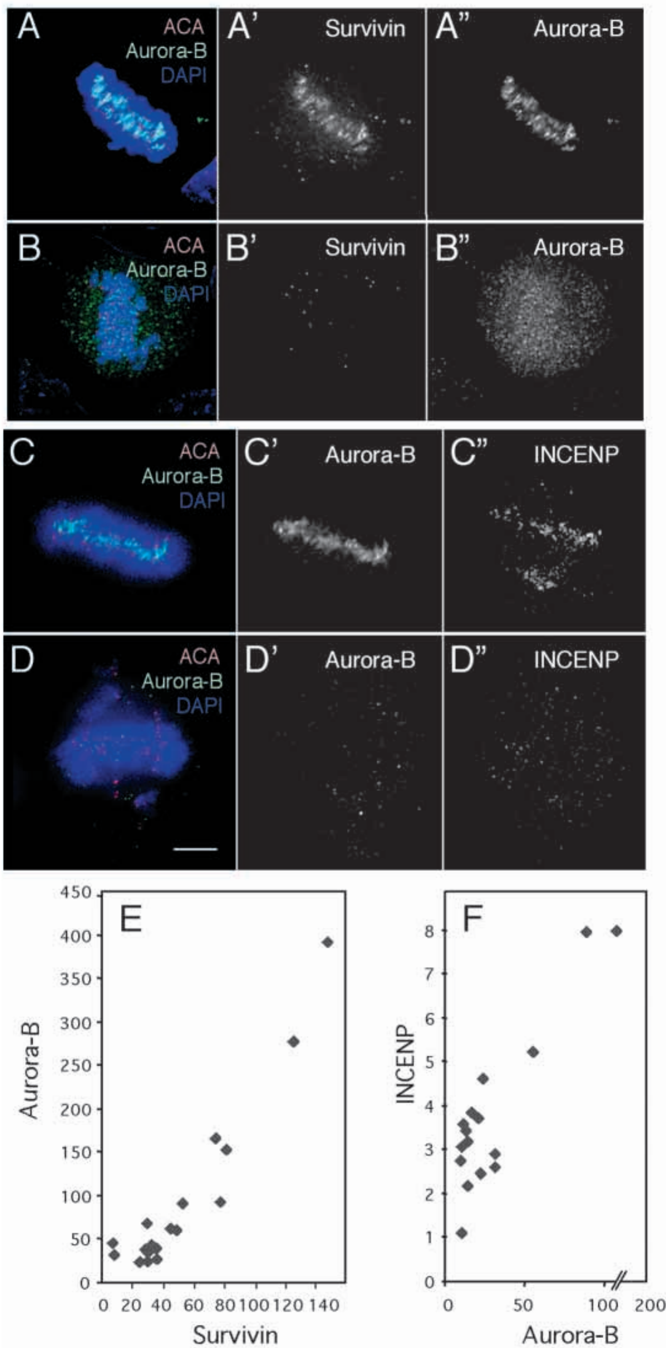

Fig. 3. Survivin depletion causes loss of Aurora-B and INCENP from the centromeres. (A-D) Survivin-siRNA-transfected cells were fixed at 60 hours and stained for Survivin, Aurora-B, INCENP and centromeres (using ACA). (A,B) Survivin versus Aurora-B. (A) Survivin-positive cell shows Survivin (A') and Aurora-B (A") at the centromeres. (B) The Survivin-depleted cell (B') shows Aurora-B diffusely localised with some residual staining on the chromosome arms (B"). Merged images do not show Survivin staining. (C,D) INCENP versus Aurora-B. (C) Survivin-positive cell, as judged by the presence of Aurora-B staining at the centromeres (green, C') shows centromeric INCENP staining (C''). (D) The Survivin-depleted cell shows no Aurora-B (green, D') or INCENP (D") at the centromeres. (E) A plot of the intensity of Survivin versus Aurora-B at centromeres revealed a linear correlation between the abundance of the two proteins. (F) A plot of the intensity of Aurora-B (to indicate Survivin depletion) versus INCENP at centromeres revealed a linear correlation between the abundance of the two proteins. (E,F) Units indicate fluorescence intensity ($\times 10^3$). Bars, 5 μm.

occasionally seen, they were also present within the Survivin-positive population (6% in Survivin-positive versus 4% in Survivin-depleted cells).

Live-cell imaging by time-lapse phase contrast microscopy confirmed that cells were delayed in mitosis following transfection with Survivin siRNA. Cells transfected with control or Survivin siRNA, were blocked in S-phase using 2 mM thymidine for 20 hours, then released into fresh medium and followed individually by time-lapse imaging (Fig. 4F). In the population transfected with control siRNA, 92% of mitotic cells (n=73) completed mitosis within 1 hour (Fig. 4G). In contrast, in the population transfected with specific siRNA, 53% of cells (n=93) remained in mitosis for longer than 1 hour (for an average of 1.7 hours in mitosis). Thus, repression of Survivin results in a delay in mitosis, and this correlates with difficulties in chromosome alignment at the metaphase plate.

Survivin depletion inhibits cytokinesis and causes a slight increase in apoptosis

Previous studies in which Survivin function was affected by the expression of dominant-negative mutants or treatment with antisense oligonucleotides have shown an increase in apoptosis and multinucleation (Li et al., 1999), albeit only a moderate increase in some cases (Kallio et al., 2001). In the present study, time-lapse imaging revealed that cells exposed to Survivin siRNA, after being delayed in mitosis for longer than control cells (see above), either flattened in interphase after failing cytokinesis or blebbed and took on an abnormal appearance. In contrast, all cells in the control population exited mitosis normally (Fig. 4F,G). We occasionally observed a very low number of Survivin-depleted cells in anaphase and telophase (Fig. 4B) but failed to detect any Survivin-depleted cells in cytokinesis (data not shown). Consistent with these observations, cultures treated with Survivin siRNA showed a gradual increase in multinucleation, culminating in a four-fold increase by 84 hours (Fig. 4E). In addition, when DNA content was measured by FACS analysis of propidium-iodide-stained cells at 84 hours, 43% of the Survivin siRNA-transfected population had a DNA content of 4N or greater compared with 22% in the control population (data not shown). Together, these observations reveal that Survivin-depleted cells eventually exit mitosis but generally fail to complete cytokinesis.

To assess the apoptotic status of cells transfected with Survivin siRNA, we performed FACS analysis using TUNEL and Annexin V labelling and confirmed the results by examining the DNA morphology microscopically (data not shown). A similar trend was observed using both methods: a moderate increase in apoptosis was detected at 72 hours after transfection with Survivin siRNA (16% higher than controls using the TUNEL assay to detect DNA fragmentation; 7% higher than controls using the Annexin V assay to detect loss of membrane asymmetry).

Together, these observations confirm that Survivin is required for the efficient completion of cytokinesis, but its requirement for the prevention of apoptosis is minor.

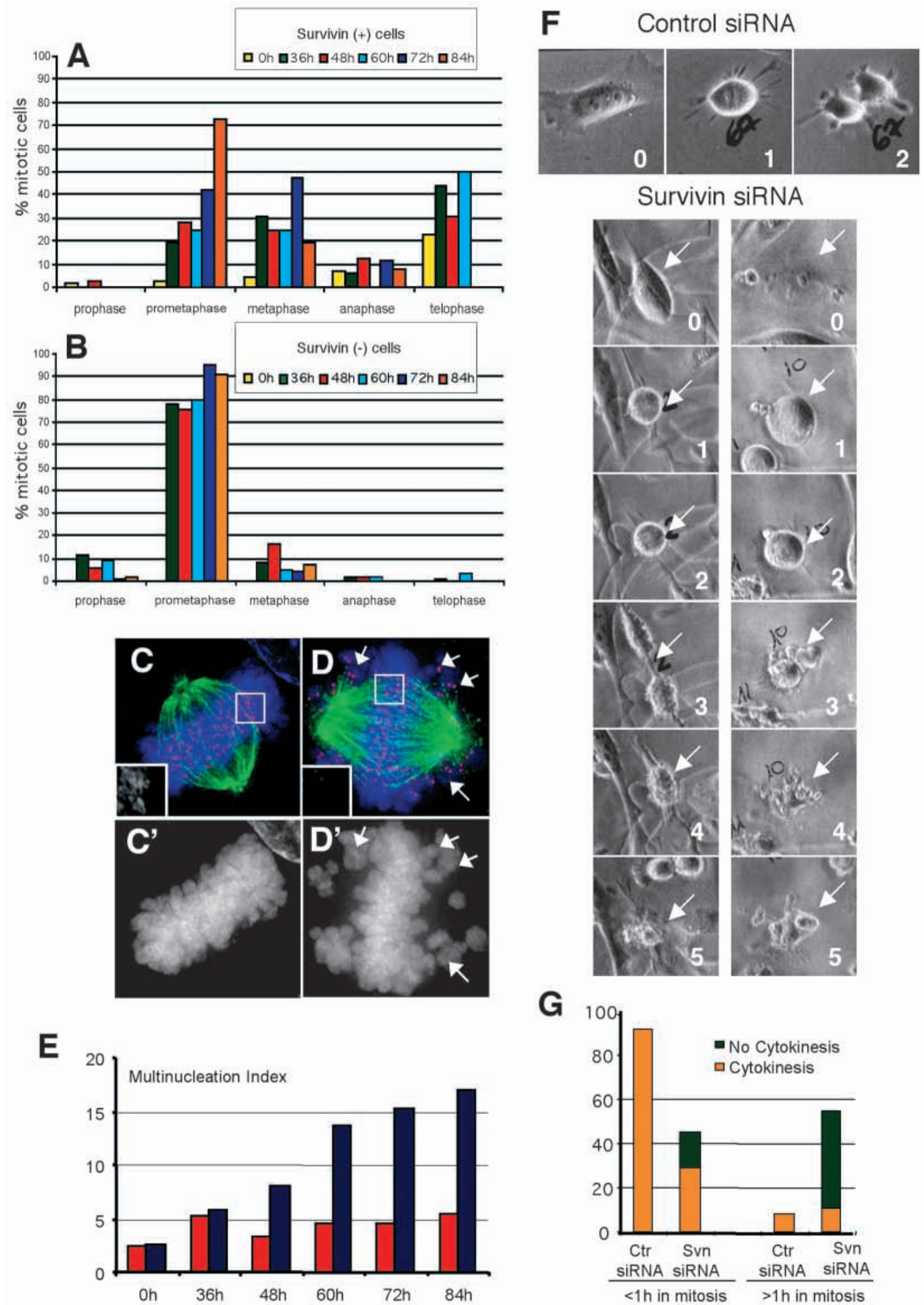

Fig. 4. Survivin RNAi causes accumulation in prometaphase, failure in cytokinesis and multinucleation. (A,B) Time course of mitotic staging of cells transfected with Survivin siRNA. Survivin-positive cells were scored in A and Survivin-depleted cells were scored in B. From 36 hours post transfection, the percentage of Survivin-depleted cells in prometaphase increases and the percentage of cells in later mitotic stages decreases. (C,D) Examples of Survivin-positive and Survivin-depleted cells in mitosis. Centromeres (red); tubulin (green); DNA (blue). Inset panels show the Survivin staining in the region indicated by the box. (C) Survivin-positive cell with all chromosomes aligned. (D) Survivin-depleted cells have misaligned chromosomes (arrows). (C',D') Chromosomes in C and D in greyscale. Bars, 5 μm. (E) Representative graph showing the percentage of multinucleated cells in cultures following transfection with control (red) versus Survivin (blue) siRNA. Multinucleation increases gradually in cultures transfected with Survivin siRNA. (F) Phase-contrast images of cells from cultures transfected with control or Survivin siRNAs, blocked in S-phase, released in fresh medium and followed by time-lapse since entering mitosis (which begins approximately 10 hours post-release from S-phase). Numbers in images indicate time (in hours) since entering mitosis. The two Survivin siRNA transfected cells shown spent >1 hour in mitosis and failed cytokinesis. (G) Histogram showing the percentages of cells from cultures transfected with control (n=73) or Survivin siRNAs (n=101) that spent up to 1 hour or more than 1 hour in mitosis before completing (yellow) or failing (green) cytokinesis.

Association of the spindle checkpoint protein BubR1 with kinetochores is unstable in Survivin-depleted cells

The increase in multinucleation at later time points following transfection with Survivin siRNA (Fig. 4E) suggested that cells were eventually overriding the spindle checkpoint and exiting mitosis despite the presence of maloriented chromosomes. We therefore examined the status of the spindle checkpoint in cells depleted of Survivin using antibodies against BubR1 and (in some experiments) Mad2. Normally, both proteins are present on kinetochores as cells enter prometaphase and are part of the

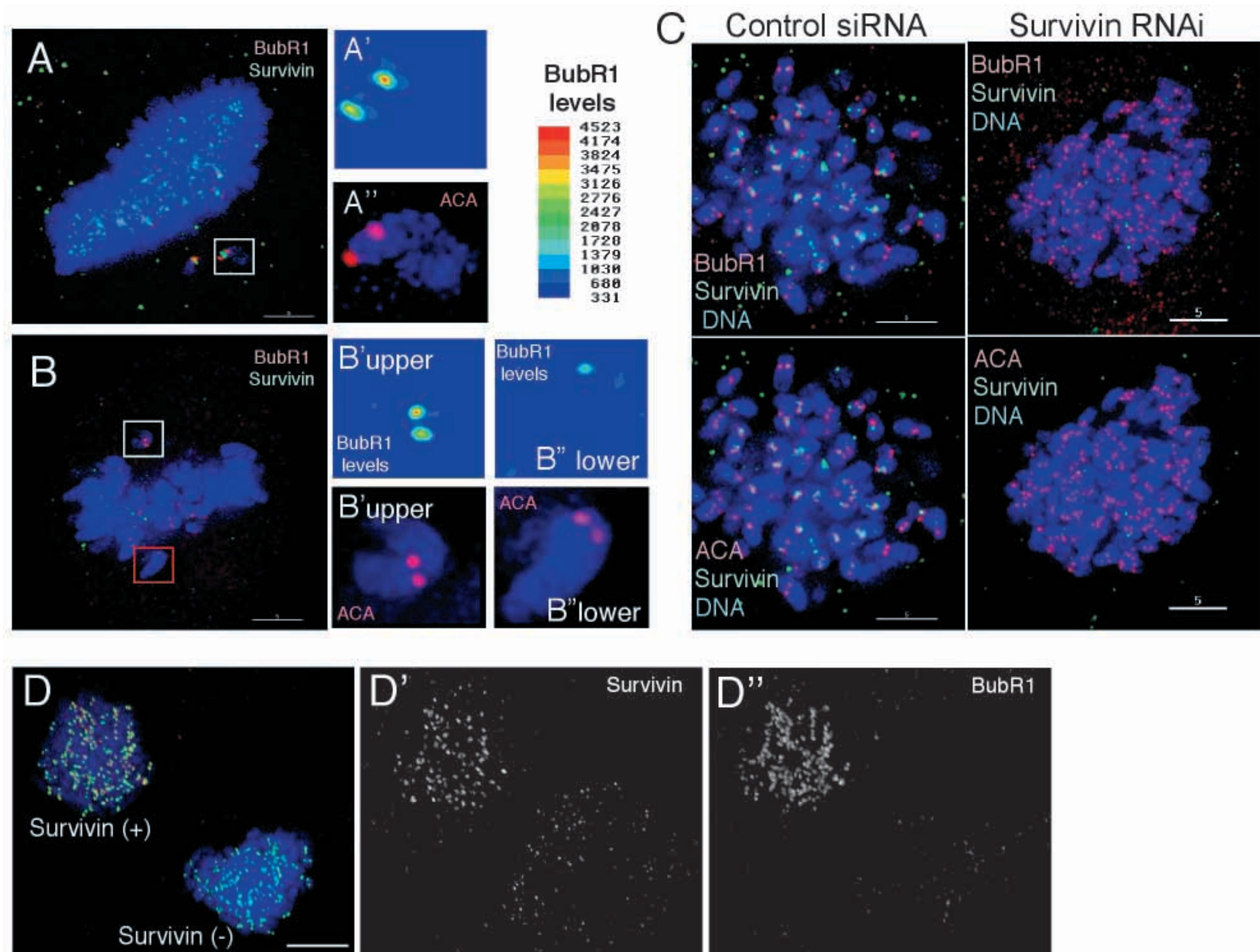

Fig. 5. Survivin depletion decreases BubR1 abundance at kinetochores of maloriented chromosomes. (A,B) Immunofluorescence images of control (A) and Survivin-depleted cells 60 hours post-transfection (B). Cells are stained for BubR1 (red), Survivin (green) and DNA (blue). Panels A'-B' show the BubR1 status of the inset regions indicated in a thermal scale where blue indicates a low signal intensity, green intermediate intensity and yellow-red high. Panels A''-B'' show enlargements of the lagging chromosomes shown in the insets stained with DAPI and ACA. Unattached chromosomes of control cells have a high BubR1 signal (A'), while both high (B', upper chromosome) and low signal intensities (B'', lower chromosome) are found on unattached chromosomes of a Survivin-depleted cell. (C) Chromosome spreads of HeLa cells following transfection with control or Survivin siRNA. Cells were treated for 2 hours with 0.1 μg/ml colcemid to depolymerize microtubules. In all panels Survivin is shown in green and DNA in blue. Top panels show BubR1 in red and lower panels show centromeres in red. All kinetochores are potentially capable of expressing high levels of BubR1 following Survivin depression. (D) Control and Survivin-depleted cells were treated with 0.1 μg/ml colcemid for 12 hours from 60 hours post transfection. A Survivin-positive and a Survivin-depleted cell are shown. BubR1 (red, D''), Survivin (greyscale, D'), centromeres (green) and DNA (blue). Survivin-depleted cell shows no BubR1 staining at the kinetochores. Bars, 5 μm.

constitutive cell cycle checkpoint that delays anaphase onset until all chromosomes are properly aligned on the spindle (Musacchio and Hardwick, 2002). Mad2 is lost from kinetochores as microtubules bind (Waters et al., 1998), and kinetochore-associated BubR1 levels fall significantly as the spindle develops tension on the kinetochore (Taylor et al., 2001; Skoufias et al., 2001).

When cells transfected with control siRNA were stained for BubR1, the kinetochores of maloriented chromosomes stained brightly, whereas the kinetochores of chromosomes aligned at the metaphase plate stained only very faintly (Fig. 5A). In contrast, in Survivin-depleted cells the BubR1 staining at kinetochores of lagging chromosomes (quantified as described in Materials and Methods) varied widely within individual cells (n=10), from negative to control levels (Fig. 5B). The presence of kinetochores with intense BubR1 staining suggests that the checkpoint pathway that monitors spindle tension can be active in Survivin-depleted cells. Therefore, Survivin is not essential for localization of BubR1 to kinetochores. However, the simultaneous presence of BubR1-negative and BubR1-positive kinetochores within the same cell implies that either (1) certain kinetochores may be defective and unable to bind BubR1 or (2) BubR1 association with kinetochores may be unstable in the absence of Survivin – either it binds once, but then fails to re-bind if spindle tension is lost, or it normally cycles on and off the kinetochore (Howell et al., 2001), and in the absence of Survivin tends to spend much more time in the off state.

To distinguish between these two possibilities, we treated cultures transfected with Survivin-siRNA with colcemid to depolymerize spindle microtubules. As shown in Fig. 5C, after 2 hours exposure to colcemid, both Survivin-positive and Survivin-depleted cells had BubR1 staining at all kinetochores. Therefore, kinetochores of chromosomes lacking detectable Survivin are capable of binding BubR1. This eliminates hypothesis one.

To test the stability of BubR1 localization to the kinetochores, cultures transfected with Survivin siRNA for 60 hours were exposed to colcemid for 3 hours, 6 hours or 12 hours, then fixed and stained for Survivin and BubR1. After 3 hours of colcemid treatment, BubR1 levels at kinetochores were similar for both Survivin-positive and Survivin-depleted cells (data not shown). However, by 12 hours in colcemid, BubR1 staining was absent from the majority of kinetochores in Survivin-depleted cells, although it remained readily detectable at kinetochores of Survivin-positive cells (Fig. 5D). Quantification of the fluorescence data revealed a linear correlation between the levels of Survivin at centromeres and BubR1 at kinetochores following 6 or 12 hours in colcemid (data not shown), indicating that Survivin is apparently required for the stable maintenance of BubR1 at kinetochores under conditions of protracted checkpoint activation.

Survivin is required for proper functioning of the spindle checkpoint in the presence of taxol

The spindle assembly checkpoint has been proposed to be bipartite, with one arm monitoring kinetochore occupancy with microtubules (Waters et al., 1998) and the other monitoring spindle tension exerted on the kinetochores (Taylor et al., 2001; Skoufias et al., 2001). The abnormalities in BubR1 staining observed in Survivin-depleted cells led us to test whether these cells were defective for one or both arms of this checkpoint pathway.

Control or Survivin siRNA-transfected cells were blocked in S-phase and released into fresh medium supplemented with 33 nM taxol for 18 hours. Taxol dampens microtubule dynamics and effectively abolishes tension within the spindle but does not necessarily prevent kinetochore-microtubule interactions (Waters et al., 1998). Cells entered mitosis ~10 hours after release from the S-phase block and were followed by time-lapse phase-contrast imaging. In the population transfected with control siRNA, 99% of the cells that entered mitosis (n=101) arrested there until the end of the experiment (Fig. 6A,C). However, in the population exposed to Survivin siRNA, 44% of the mitotics (n=103) exited mitosis as judged by cell re-spreading and formation of multiple micronuclei (Fig. 6A,C). That these cells had exited mitosis was subsequently confirmed by staining with DAPI (data not shown). By contrast, when cells were released from the S-phase block into medium supplemented with 0.1 μg/ml nocodazole, a microtubule depolymerizing agent, both control and Survivin-siRNA-transfected populations arrested efficiently in mitosis (Fig. 6B,C). These results show that repression of Survivin compromises checkpoint function following exposure to taxol but not nocodazole.

To further characterise the checkpoint response after Survivin repression, live cells were followed in the presence of nocodazole or taxol and then subsequently fixed and immunostained for BubR1 and Mad2. Mitotic cells in cultures transfected with control siRNA showed a normal response to both drugs. Cells in taxol (kinetochores occupied with microtubules, but spindle tension defective) stained brightly for BubR1 at kinetochores, but only a few stained for Mad2 (most of the kinetochores are attached to microtubules) (Fig. 6D). Cells in nocodazole (kinetochores unoccupied and no tension) stained brightly for both BubR1 and Mad2 at kinetochores (Fig. 6F). In contrast, kinetochores of Survivin-depleted cells in taxol either completely lacked or showed a very decreased staining for both BubR1 and Mad2 (Fig. 6E). Furthermore, in the presence of nocodazole, Survivin-depleted cells also showed no or very low staining for BubR1, but Mad2 remained bright at the kinetochores (Fig. 6G).

In summary, these data, which are summarized in Table 1, indicate that Survivin is required for the function of the spindle checkpoint in response to taxol treatment but not in response to nocodazole.

Table 1. Summary of immunofluorescence results

Drug	Survivin functional*	BubR1	Mad2	Mitotic arrest?
Nocodazole	+	+	+	+
Nocodazole	–	–	+	+
Taxol	+	+	–	+
Taxol	–	–	–	–

*Cells were stained for Aurora-B/AIM-1. As shown in Fig. 3, levels of Survivin correlate directly with levels of Aurora-B; therefore centromeres negative for Aurora-B were judged to be negative for Survivin function.

Discussion

Transfection of HeLa cells with an siRNA designed to eliminate all known forms of human Survivin (Mahotka et al., 1999; Rodriguez et al., 2002) led to the loss of detectable Survivin as assayed by immunoblotting and indirect immunofluorescence microscopy using an antibody recently shown to recognise all known forms of the protein (Fortugno et al., 2002). This correlated with a cessation of population growth 48-60 hours post-transfection and was accompanied by an accumulation of prometaphase cells with bipolar spindles and misaligned chromosomes.

Recently, microinjection of polyclonal antibodies that recognise all known forms of Survivin into HeLa cells was reported to result in similar chromosome congression defects (Giodini et al., 2002). However, earlier studies had suggested that interference with Survivin function compromised the ability of cells to form a normal bipolar spindle. In HeLa cells (the cell type used here), a significant increase in multipolar spindles was reported after exposure of cells to a dominant-negative Survivin construct (C84A) or antisense oligonucleotides (Li et al., 1999) and, shown more recently, after microinjection of Survivin antibodies (Fortugno et al., 2002). Multipolar spindles were also observed after interference with Survivin function in other systems, for example, early embryonic cells derived from Survivin-knockout mice (Uren et al., 2000). It is worth noting that in the present study, although we detected an approximately 10% increase in multinucleated cells over control levels, no significant increase in the percentage of multipolar mitosis was observed.

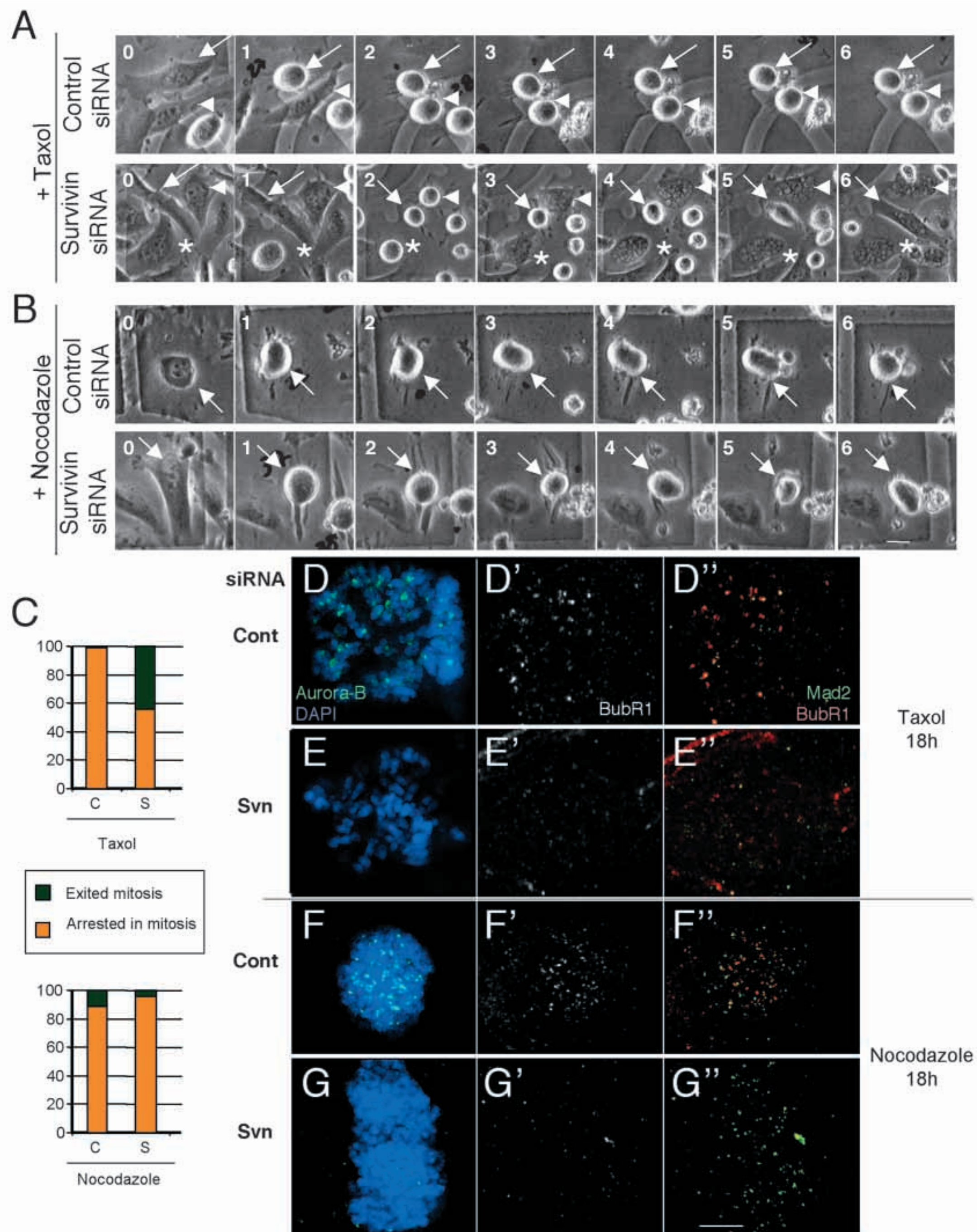

Fig. 6. Survivin is required for checkpoint arrest in response to taxol. (A,B) Phase-contrast images of cells from cultures transfected with control or Survivin siRNAs that were blocked in S-phase, released in medium supplemented with 33 nM taxol (A) or 0.1 µg/ml nocodazole (B) and followed every hour after entering mitosis for 8 hours. Arrows, arrowheads or asterisks indicate individual cells of interest. (C) Histogram showing the percentages of cells from cultures transfected with control (C) or Survivin siRNAs (S) that either arrested in mitosis (yellow) or exited into interphase (green) in the above experiment. (D-G) Immunofluorescence staining of cells treated as in Fig. 6A,B, stained for Aurora-B (green), DNA (blue), BubR1 (greyscale in D',E',F',G', red in D'',E'',F'',G'') and Mad2 (green in D'',E'',F'',G''). Survivin-depleted cells (as judged by Aurora-B staining) show no little or no staining for BubR1 or Mad2 at the kinetochores in the presence of taxol. In the presence of nocodazole, Mad2 staining is present at the kinetochores of both control and Survivin-depleted cells, whereas BubR1 is either greatly depleted or absent. Bar, 5 µm.

Time-lapse imaging revealed that transfection with Survivin siRNA caused a mitotic delay and failure of cytokinesis. The net result was a significant increase in the number of polyploid and multinucleated cells in the population. Furthermore, we found that levels of apoptosis rose only slightly, confirming that Survivin functions predominantly in mitotic progression as opposed to inhibition of cell death.

These observations confirm that Survivin is required for chromosome biorientation and completion of cytokinesis in HeLa cells. The reason for the defects in cytokinesis induced

by repression of Survivin is not known, but one possibility is that Survivin-depleted cells exit mitosis with maloriented chromosomes and that these are trapped in the intercellular bridge, where they block the completion of abscission. Consistent with this view, we have observed that Survivin-depleted cells have defects in the spindle assembly checkpoint (discussed further below).

Survivin is not a mitotic spindle associated protein prior to anaphase

Survivin was originally described as a mitotic-spindle-associated protein (Li et al., 1998; Li et al., 1999). However we and others have subsequently found that Survivin behaves like a chromosomal passenger protein in human (Skoufias et al., 2000; Wheatley et al., 2001a; Jiang et al., 2001) and murine cells (Uren et al., 2000). Recent papers raised the possibility that there are two populations of Survivin (Fortugno et al., 2002) or that different cell types had different localizations of the protein (Jiang et al., 2001). At the centre of this controversy is a monoclonal antibody, 8E2, which strongly stains the entire spindle in metaphase cells (Fig. 2C). In this study we found that the pattern of 8E2 staining remained unaltered when Survivin was depleted from HeLa cells (Fig. 2D). Although this monoclonal antibody does recognise human Survivin in vitro (Wheatley et al., 2001a), the persistence of spindle staining after depletion of all known Survivin isoforms demonstrates that 8E2 is likely to also recognise an additional unknown microtubule-associated protein in mitotic HeLa cells.

Mutual dependence of Survivin, Aurora-B and INCENP for localization at centromeres

Aurora-B and INCENP fail to concentrate at centromeres in prometaphase cells depleted of Survivin. Because a dominant-negative form of INCENP causes the mislocalization of Aurora-B and Survivin in HeLa cells (Adams et al., 2000; Wheatley et al., 2001a), it appears that these three proteins are mutually dependent on each other for localization to centromeres. In *C. elegans*, the homologues of Survivin [BIR1 (Fraser et al., 1999)], Aurora-B (AIR-2) and INCENP (ICP1) also appear to interact with one another, and the localization of AIR-2 is dependent upon ICP-1 (Kaitna et al., 2000) and BIR1 (Speliotes et al., 2000). In addition, corroborating evidence regarding the relationship(s) between these three chromosome passengers is also emerging in flies (Adams et al., 2001c), frog eggs (Bolton et al., 2002) and yeast (Yoon and Carbon, 1999; Rajagopalan and Balasubramanian, 2002; Cheeseman et al., 2002). One possible explanation for these data is that a stable complex between Survivin, Aurora-B and INCENP is required to target this group of proteins to the centromeres. However, the persistence of such a complex throughout mitosis has yet to be demonstrated directly.

Survivin is required for the efficient localization of BubR1 to kinetochores and a robust spindle checkpoint response in the presence of taxol

As cells enter mitosis, kinetochores constitutively express signals that delay the activation of the APC/C (anaphase-promoting complex/cyclosome) and onset of anaphase (Pines and Rieder, 2001). These signals are gradually extinguished as sister kinetochores capture microtubules from opposite spindle poles and tension develops throughout the spindle. This signalling pathway, termed the spindle assembly checkpoint, has been proposed to involve two separate components. The BubR1 arm of the pathway has been suggested to monitor spindle tension (Taylor et al., 2001; Skoufias et al., 2001), whereas the Mad2 pathway monitors microtubule attachment at the kinetochore (Waters et al., 1998). However, a recent study has questioned whether these represent two completely independent, parallel pathways or a single pathway that depends on both BubR1 and Mad2 (Shannon et al., 2002).

The present study has shown conclusively that Survivin-depleted cells exhibit important differences from control cells in the function of their spindle assembly checkpoint when exposed to agents that perturb microtubule behaviour. We found that in Survivin-depleted cells, some kinetochores of maloriented chromosomes expressed high levels of BubR1 protein, whereas kinetochores of other maloriented chromosomes did not. Importantly, kinetochores of Survivin-depleted chromosomes accumulated BubR1 normally during a short colcemid (2 hours) treatment, indicating that Survivin is not required for BubR1 binding to the kinetochore upon activation of the spindle assembly checkpoint. Instead, we found that Survivin is required for the maintenance of BubR1 at kinetochores during persistent activation of the checkpoint. This is consistent with the results of a recent study (Kallio et al., 2001) in which it was reported that the phospho-epitope, 3F3/2, which identifies kinetochores that are not under tension during prometaphase (Nicklas et al., 1995), was precociously lost from centromeres when cells were microinjected with Survivin-specific antibodies.

Normally, in the presence of taxol, kinetochores have elevated levels of BubR1 and are negative for Mad2. In the presence of nocodazole both proteins are normally present at elevated levels. Although recent experiments have revealed that it is dangerous to draw conclusions about the activity of the spindle assembly checkpoint based solely on the level of kinetochore staining with anti-Mad2 (Martin-Lluesma et al., 2002), in the case of Survivin siRNA treatment, the observed pattern of kinetochore association of BubR1 and Mad2 is able to explain the response of the cells to persistent checkpoint activation.

We have found that following Survivin RNAi, BubR1 accumulation at kinetochores is unstable in the presence of either taxol or nocodazole. Therefore, in the presence of taxol, kinetochores lack elevated levels of both BubR1 and Mad2. As a result, Survivin RNAi abrogates the ability of cells to sustain a prolonged mitotic arrest in the presence of taxol. In contrast, Survivin RNAi has no effect on the ability of kinetochores to retain high levels of Mad2 protein. Thus, in the presence of nocodazole or colcemid, kinetochores are BubR1 negative but Mad2 positive, and cells are able to sustain a prolonged mitotic arrest.

Kinetochores that express elevated levels of either BubR1 or Mad2 alone appear to be able to generate a 'wait anaphase' signal and activate the spindle assembly checkpoint. The former are seen normally in the presence of taxol, and the latter are described here for the first time. It thus appears that either BubR1 or Mad2 and their downstream effectors are sufficient to activate the checkpoint.

We note that the major conclusions described above, and particularly the requirement of Survivin for a robust checkpoint

response to taxol but not nocodazole, are consistent with the results of an independent study in which Survivin silencing was achieved using a plasmid vector in several different cell lines (Lens et al., 2003).

Importantly, our data should not be interpreted as indicating that Survivin is directly required for the detection of spindle tension. Rather, they indicate that Survivin is required for stable maintenance of elevated levels of BubR1 at kinetochores. The preferential sensitivity of the taxol-induced checkpoint to loss of Survivin reflects more the behaviour of Mad2 than any specific alteration in the behaviour of BubR1. The result may appear to be selective for one arm of the checkpoint pathway, but the actual effect on BubR1 is not selective. Thus, although the data presented here appear to support the emerging view of a dual checkpoint pathway, where one arm detects kinetochore occupancy, and the other detects spindle tension (Taylor et al., 2001; Skoufias et al., 2001), whether or not there is a dual input to the spindle assembly checkpoint pathway remains an important question for future investigations.

Concluding remarks

As Survivin presumably functions as a targeting or stimulatory factor for the Aurora-B protein kinase, these results in effect confirm the remarkable conservation of the checkpoint pathway. Studies in budding yeast have shown that the Ipl-1 kinase (yeast homologue of Aurora-B) is required for the spindle checkpoint in response to a loss of spindle tension but not in response to the loss of microtubule binding to kinetochores (Biggins and Murray, 2001). Thus, not only the core checkpoint machinery but also the modulation of this machinery by the Aurora-B kinase is conserved from yeast to man. Aurora-B regulates kinetochore-microtubule interactions in budding yeast (Tanaka et al., 2002), and it phosphorylates components of the Dam1 complex (Cheeseman et al., 2002), whose human homologues have yet to be identified. It now becomes a matter of some urgency to identify the kinetochore protein(s) whose modification by Aurora-B is required for the stable association of BubR1 with kinetochores under conditions of persistent checkpoint activation.

It is likely that one common thread underlying the multiple phenotypes seen in the present study is the action of the Aurora-B kinase. The search for relevant substrates of this kinase is ongoing and likely to yield many candidates. However, in addition to targeting and activating Aurora-B, which is becoming ever more recognised as an essential facilitator of mitotic events (Adams et al., 2001a), it is also possible that Survivin and INCENP have other roles in mitotic progression. Thus, this remains an area of growing excitement and important discovery.

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