Survivin and plk1 kinase are important mediators of cell survival that are required for chromosome alignment, cytokinesis, and protection from apoptosis. Interference with either survivin or plk1 activity manifests many similar outcomes: prometaphase delay/arrest, multinucleation and increased apoptosis. Moreover, the expression of both survivin and plk1 is deregulated in cancer. Given these similarities, we speculated that these two proteins may cooperate during mitosis, and/or in cell death pathways. Here we report that survivin and plk1 interact during mitosis, and that plk1 phosphorylates survivin at serine 20. Importantly we find that overexpression of a non-phosphorylatable version, S20A, is unable to correct chromosomes connected to the spindle in a syntelic manner during prometaphase, and allows cells harbouing these maloriented chromosomes to enter anaphase, evading the spindle tension checkpoint. By contrast, the constitutive phosphomimic, S20D, completes congression and division ahead of schedule, and, unlike S20A, is able to support proliferation in the absence of the endogenous protein. Despite the importance of this residue in mitosis, its mutation does not appear to affect survivin’s anti-apoptotic activity, in response to TRAIL. Together, these data suggest that phosphorylation of survivin at S20 by plk1 kinase, is essential for accurate chromosome alignment and cell proliferation but is dispensable for its anti-apoptotic activity in cancer cells.

Survivin is a protein with multiple functions, whose expression is deregulated in cancer. It is best known for its participation in the chromosomal passenger protein (CPP) complex during mitosis, and its ability to inhibit apoptosis (reviewed in (1-3)). During mitosis survivin is regulated by the kinases, aurora-B and cdk1. Mutation of the aurora-B phosphorylation site at T117 (4), or treatment with an aurora-B inhibitor alters survivin’s affinity for centromeres and interferes with the error correction process facilitated by CPPs that ensures proper alignment of chromosomes at the metaphase plate (5-7). Furthermore, data from a constitutive phosphomimic suggests that phosphorylation of survivin by aurora-B prevents the completion of cytokinesis, inferring a critical requirement for dephosphorylation of this site (7). Cdk1 phosphorylates survivin at threonine 34 (T34) in its BIR domain (8). Mutational analysis has shown that expression of a T34 phosphomimic, T34E, greatly reduces the rate of cell proliferation and cannot support cell division in the absence of the endogenous protein, while expression of the non-phosphorylatable counterpart, T34A, supports cell growth (9,10). Intriguingly T34A sensitises cells to apoptotic stimuli, and is being explored as a potential therapeutic tool (2,11), while T34E potently inhibits cell death (9,12,13). Thus phosphorylation by cdk1 is one means of separating survivin’s mitotic and anti-apoptotic roles.

Polo-like kinase 1 (plk1) is also an essential, multitasking protein, whose expression is deregulated in cancer. First identified in Drosophila (14), plk1 regulates mitotic entry, centrosome separation, spindle assembly, chromosome alignment, APC/C activation, cytokinesis, and has been implicated as a mediator of apoptosis (15). In cultured mammalian cells polo disruption has been achieved using a number of different techniques including chemical genetics (16,17), small molecule inhibition (18-21) and RNAi (22,23). As expected for a protein with many roles, its loss has pleiotropic effects including the generation of monopolar spindles, polyplody,
and increased apoptosis. Although the majority of plk1 is centrosomal in early mitosis, a
subpopulation associates with the kinetochores (24), and has been implicated in mediating the
spindle checkpoint (22,23). Mad2 and BubR1 are checkpoint proteins that are recruited to the
kinetochores of chromosomes that are not properly attached to the spindle. Mad2 is
recruited due to the absence of microtubule attachments, while BubR1 is recruited when
paired kinetochores are not under tension. Interestingly, treatment of plk1 or survivin-
depleted cells with microtubule poisons has suggested that plk1 stabilises Mad2 recruitment
at kinetochores (22), while survivin stabilises BubR1 at these sites (25,26). Supporting this
notion, simultaneous depletion of survivin and plk1 eliminates both spindle checkpoint signals
and consequently cells exit mitosis inappropriately and undergo mitotic catastrophe
(22). However, Matsumura et al., (23), recently reported that plk1 interacts directly with BubR1,
and that phosphorylation of BubR1 by plk1 is required for correct chromosome orientation
during prometaphase, but not for its recruitment to kinetochores, nor for spindle checkpoint
activation. Thus although plk1 and survivin may have complementary roles in the maintenance of
the spindle checkpoint, direct links between plk1 and BubR1 also exist that facilitate chromosome
bi-orientation. In cells that enter anaphase normally plk1 is found at the central spindle and
midbody, where it colocalises with the CPPs and is required to facilitate cytokinesis in
communication with the microtubule organisers, MKLP1, MKLP2 and PRC1, and the RhoA
signalling cascade (27-29).

In the present study we report that survivin and plk1 kinase interact during mitosis,
and that survivin is a plk1 substrate. We identify S20 as a principle target of plk1 within the
survivin protein, and discover that inhibiting phosphorylation at this site interferes with the
correction of syntelically attached chromosomes. Inhibiting phosphorylation at this site also
prevents cell proliferation in the absence of the endogenous protein, but does not affect cellular
response to an apoptotic stimulus. We conclude that phosphorylation of survivin by plk1s
essential to prevent aneuploidy caused by maloriented chromosomes. Further, these data
demonstrate a second phosphorylation event, distinct from that of cdk1, capable of divorcing
survivin’s mitotic and anti-apoptotic roles.

Experimental Procedures.
Unless otherwise stated all cell culture reagents were from Invitrogen, and general chemicals
from Sigma-Aldrich.

Molecular Biology.
Site directed mutagenesis was carried out by
Quik Change Site Directed mutagenesis (Stratagene) using wild type survivin cDNA with a
silent mutation in its RNAi targeting region, cloned in pBluescript, as template, see (30). Once
sequences were verified, the constructs were cut and pasted into pcDNA3.1 with a C-
terminal GFP tag for expression in mammalian
cells, or into pGEX4T1 for NH2-terminal GST
tagging and recombinant expression.

Wild type full length plk1 cDNA was amplified from IMAGE clone 2822226 (Au2-e5;
Geneservice) using the 5’-primer GCTTGAAATTCATGAGTGCTGCAGT, and the
3’-primer GCTTCTCGAGTTAGGAGGCCTTCGA,
containing an EcoRI and Xho1 site respectively
for subsequent cloning procedures. The region
encoding the polo binding domain (PBD) and
the polo kinase domains (KinD) were PCR’d from
the full length template using similar flanking
enzymes and appropriately designed primers.

Cell Culture and Drug Treatments.
U2OS cells were maintained at 37°C with 5%
CO2 in DMEM/ 10% FCS (PAA), with
penicillin-streptomycin, and fungizome. U2OS
cells stably expressing the various forms of
survivin, were established by FuGene 6-mediated
transfection with 1-2 µg of the relevant
pcDNA3.1 constructs. Twenty four hours post-
transfection cells were exposed to 500 µg/ml
G418, and 7-10 days later single GFP-expressing
colonies were selected. Pools consisting of a
minimum of four separate clones were used for
analysis. Cells were arrested in prometaphase by
overnight incubation in 0.2 µg/ml colcemid or 2
µM dimethylastron (www.axxora.com), and
released by extensive washing in PBS (room
temperature) and reincubation in complete
DMEM at 37°C. To inhibit Plk1 activity cells
were incubated overnight with 50 nM BI 2536
(Tocris). When imaging cells live, regular
DMEM was substituted for CO2 independent
medium without phenol red.

Recombinant Protein Expression.
For bacterial expression of recombinant survivin,
polo kinase and their variants, pGEX4T1 vectors
encoding the relevant cDNAs were transformed into BL21 cells. Protein expression was induced by the addition of 0.5 mM IPTG for 3 h at 30°C, and lysates prepared as in (30). Recombinant GST tagged proteins were then bound to glutathione sepharose 4B beads (G.E. Healthcare) and eluted in 50 mM Tris-HCl (pH 8) containing 10 mM glutathione. When stated, GST was cleaved off the recombinant protein/beads using thrombin, which was then removed with benzamidine agarose.

For GST-pull down experiments, the GST-tagged proteins bound to beads were incubated with an in vitro translated “partner” protein expressed from pcDNA or pBluescript using a T7-T7 transcription-translation kit (Promega) and 35S methionine (Perkin-Elmer) as tracer, see (30) for detailed methods.

**Fluorescence Microscopy.**

To localise plk1 kinase, cells were fixed with 4% formaldehyde in PBS for 5 minutes, washed with PBS, permeabilised with 0.15% triton for 2 minutes, and blocked with 1% BSA in PBS for 15 minutes and immunoprobed with anti-plk1 (AbCam, Ab14209; 1/100), anti-CENPC (AbCam, Ab50974, 1/250), anti-auroa-B (AbCam, Ab2254, 1/1000), anti-borealin (polyclonal, in-house), or anti-BubR1 (1/250, gift from S.S.Taylor, Manchester) antibodies, followed by texas-red anti-rabbit, anti-sheep or anti-mouse antibodies as appropriate (1/200, Vector Labs). For tubulin localisation cells were probed with 1/200 anti-alpha tubulin (B512) and texas-red anti-mouse (1/200, Vector Labs).

To visualise F-actin, formaldehyde-triton fixed cells were incubated for 30 minutes at room temperature with 200 nM rhodamine-phalloidin prepared in PBS. All cell preparations were counterstained with the DNA stain DAPI upon mounting in Vectashield (Vector Labs). Fixed cells were viewed using an inverted fluorescence Olympus microscope fitted with a 60 x (NA 1.4), or 100 x (NA 1.3) oil immersion objective, and images captured using DeltaVision software (Applied Precision). 2D projections were generated from deconvolved Z-stacks and images prepared using Adobe Photoshop.

High resolution live cell imaging was performed using an Olympus based personal Delta Vision workstation at 100 x (NA 1.4, oil). A Z-sweep of 40, 0.3 µm sections was acquired at each time point every 2 minutes, using both DIC and GFP optics. Subsequent offline images preparation was carried out using Velocity software (www.improvision.com), and finalised with Adobe Photoshop. Images in Figure 5H were acquired using a Leica DMIRB, fitted with x40 oil immersion lens, using Open Lab software (www.improvision.com).

**Immunoprocesses.**

For immunoprecipitation whole cell lysates were prepared from 3 x 10^6 cells by 1h incubation at 4°C in NP40 buffer (50 mM Tris-HCl (pH 8), 150 mM NaCl, 10 mM EDTA, 1% NP40). After clearing by centrifugation the supernatant was then incubated with rotation for 2 h with 2-4 µg antibody, then 25 µl protein G beads were added for a further 2 h (or overnight) at 4°C. To IP plk1 a mixed population of anti-plk1 monoclonal antibodies was used (AbCam 14210; 2 µg/ 3 x 10^6 cells, and to IP GFP, an in-house polyclonal rabbit antibody was used at 4 µg/ 3 x 10^6 cells). Beads were washed with NP40 buffer and proteins boiled off the beads with 5 x Laemmli sample buffer.

Standard procedures were used for SDS-PAGE and immunoblotting with 0.22 µm nitrocellulose membrane. ECL–plus (G.E. Healthcare), X-ray film (G.E.Healthcare) and a Storm phosphomager were used to detect signals. After SDS-PAGE and transfer to nitrocellulose membrane, immunoblots were probed with polyclonal anti-survivin antibodies (1/2000, in-house); anti-plk1 kinase (rabbit, AbCam 14209, 1/100), anti-GFP (mouse monoclonal 3E1, 1/500, CR-UK); anti-cyclin B1 (1/500; BD Biosciences); anti-actin (1/5000); anti-GFP, anti-phosphorylated histone H3 (Upstate, 1/1000), anti-tubulin (B512, 1/2000) or anti-GST (AbCam, Ab9085; 1/500). HRP-conjugated secondary antibodies (DAKO) were diluted 1/2000 in 3% milk.

For Far Western analysis, 5 µg of untagged recombinant survivin was phosphorylated, subjected to SDS-PAGE then transferred to Hybond-C nitrocellulose membrane. The membrane was then blocked in AC buffer (20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.5 mM EDTA, 0.1% Tween 20), and 2% milk for 1h. Next the membrane was incubated overnight at 4°C with 5 µg/ml of recombinant GST-tagged polo binding domain in AC buffer with 2% milk and 1 mM DTT. To assess GST-PBD binding the membrane was probed with polyclonal goat anti-GST antibodies (AbCam, Ab6613, 1/5000) for 2h and detected using standard HRP/ ECL methods, as above.
In vitro kinase assays.
Recombinant Plk1 kinase (Cell Signalling) or plk1 immunoprecipitated with protein-G beads (CR-UK) and anti-plk1 antibodies (mixed monoclonal mouse antibodies, AbCam, Ab14210, 2 µg/mg cell extract) were used for in vitro kinase assays. Two microlitres of recombinant plk1 kinase, or 10 µl of plk1-protein G beads were incubated with 2 µg of recombinant substrate in plk1 kinase buffer (25 mM Tris-HCl, pH7.5, 5 mM MgCl2, 50 mM NaCl, 1 mM DTT, 0.5 mM EDTA) and reaction started by the addition of 1 µl 2 mM ATP and 0.5 µl 32P-γ ATP (5 µCi per reaction; Perkin Elmer). The reaction (final volume 20 µl) was incubated for 10-40 minutes at 37°C and stopped with sample buffer. To inhibit plk1 activity in vitro, 500 nM GW843682X (Tocris) was added to the reaction.

In vitro phosphorylation with other recombinant kinases (for Figure 2E) were performed similarly using purified cdk1 (Cell Signalling), aurora-B-INCENP (IN-box; a gift from Dr. P. Eyers), or CK2 (a gift from Prof. E. Pinna).

siRNA.
Endogenous survivin was eliminated from U2OS cells using a double pulse procedure with siRNA oligonucleotides directed against nucleotides 54-65 (Carvalho et al., 2003). 5 x 10^6 cells were reverse transfected with 3 pmoles survivin siRNA using Hyperfect (Qiagen) in antibiotic-free DMEM in 24 well plates. Twenty-four hours later cells were exposed to a further pulse of 3 pmoles siRNA. Thereafter, cell proliferation was monitored at 24h intervals using a haemocytometer and trypan blue exclusion. Other analyses were carried out 48h – 96 h after the first pulse.

FACS Profiling.
Cells were harvested in ice cold PBS, and fixed with 70% ethanol (-20°C) for a minimum of 2h. They were then washed with PBS and treated with 100 µg/ml RNase and stained with 100 µg/ml propidium iodide for at least 15 minutes at 25°C. Samples were analysed using a FACS Canto (Becton Dickinson).

Apoptosis Assay.
To assess the ability of cells to inhibit apoptosis, 10^5 cells were seeded into 24 well plates on day 0, and the following day apoptosis was induced by the addition of 250 ng/ml recombinant human TRAIL (Peprotech) for 30, 60, 90 or 120 minutes, as indicated. Cells were then lysed in 150 µl of mammalian protein extraction reagent (MPER; Perbio) with 1 mM EDTA, in the presence of 1 µg/ml each of the protease inhibitors pepstatin A and AEBSF. To assess apoptotic activity, 40 µl of each lysate (in triplicate) was incubated per well of a 96 well plate with 200 µl caspase assay buffer (20 mM Tris (pH 7.5), 10% glycerol, 2 mM DTT) and 4 ng of the caspase-3/7 specific substrate, Ac-DEVD-AMC (Biomol). After incubation at 37°C for a minimum of 1h caspase activity was assessed fluorogenically using a SpectraMax Gemini Spectrofluorometer set at 380 nM (excitation) and 440 nM (emission).

Results.
Survivin and plk1 associate in vivo during mitosis.
To begin our investigation, we first used immunolocalisation to determine whether plk1 colocalised with survivin-GFP in our system (Figure 1A). U2OS cells stably expressing survivin-GFP, probed with antibodies to plk1 revealed that during early mitosis (prometaphase and metaphase), although the majority of plk1 kinase was present on the centrosomes, a subpopulation localised to the kinetochores adjacent to the survivin-GFP at the centromeres. Thereafter all plk1 kinase colocalised with survivin-GFP, decorating the midzone microtubules during anaphase and midbody during cytokinesis.

Next we asked whether the two proteins associate in vivo. Reciprocal co-immunoprecipitations were performed using plk1 or GFP antibodies in U2OS cells cotransfected with pcDNA vectors expressing GFP and plk1 or survivin-GFP and plk1 (Figure 1B). When plk1 was immunoprecipitated an abundant band was visible in the survivin-GFP lane, but not in the GFP control lane (Figure 1B, left panel). Conversely, when antibodies to GFP were used, a strong plk1 band co-immunoprecipitated with survivin-GFP expressing cells, but not with the GFP control (Figure 1B, right panel). Thus survivin and plk1 colocalise and co-immunoprecipitate in U2OS cells, which both confirms the recent findings of Feng et al., (31), and validates our system.

As survivin-GFP and plk1 kinase show greater colocalisation during anaphase, telophase and cytokinesis than in prometaphase or...
Regulation of survivin by plk1.

metaphase, we next asked whether the ability of survivin-GFP to associate with plk1 kinase increased as cells progressed through mitosis. The plk1 immunoprecipitation experiment was repeated using extracts prepared from survivin-GFP expressing U2OS cells, after transient transfection with cDNA to plk1 kinase at 30 minutes intervals post-release from a nocodazole induced prometaphase arrest. As shown in Figure 1C survivin-GFP coimmunoprecipitated with ectopic plk1 kinase at all time points, and its association grew stronger as cells exited mitosis, as indicated by the reduction in cyclin B1 expression in the accompanying whole cell extracts. When the same experiment was carried out in U2OS cells expressing no exogenous survivin, the overexpressed plk1 kinase immunoprecipitated endogenous survivin, and displayed a similar profile of association during mitosis (Figure 1D). We also noted that this increased association occurred despite an overall decline in endogenous survivin levels, which occurs as cells exit mitosis. Further immunoprecipitations revealed that the endogenous forms also co-associate (see Figure 5H).

Survivin and plk1 kinase interact directly in vitro.

To assess whether the interaction between survivin and plk1 was direct, we turned to in vitro analysis. GST, GST-survivin or various NH2 and C-terminally truncated forms were incubated with in vitro 35S-labelled full length plk1 kinase. As shown in Figure 2A, plk1 bound to full length survivin and more strongly to the C-terminally truncated forms of survivin, 1-90, and 1-115. By contrast binding to the NH2-terminal truncations, 90-142 and 98-142, was comparable to the GST control. Thus we conclude that the interaction with plk1 is mediated by the NH2 end of survivin. The reciprocal experiment was then performed to map the part(s) of plk1 that interact with survivin, or the NH2 90 amino acids of survivin (1-90). GST, GST-plk1, the NH2 half containing the kinase domain, GST-KD, or the C-terminal half with the polo binding domain, GST-PBD, was incubated with in vitro translated 35S-labelled full length survivin, or survivin 1-90 (Figure 2B). Here survivin bound to GST-plk1 but not to GST alone, confirming that the two proteins interact directly in vitro. Survivin interacted with both the kinase and the polo binding domains, suggesting that it can bind plk1 via two distinct sites, while the NH2 terminal 1-90 bound most tightly to the GST-PBD region.

Plk1 phosphorylates survivin at S20.

Having established that survivin can bind plk1 both in vivo and in vitro, we then asked whether survivin is a plk1 substrate. In vitro kinase assays were performed with either recombinant or immunoprecipitated plk1 (Figure 3 and data not shown). Plk1 phosphorylated itself (Figure 3A, plk1S), full length GST-survivin (Figure 3A, lanes 3 & 4, SvnS) but not the GST control (Figure 3A, lanes 1 & 2). Of all the truncations tested (see Table 1), the longest version to show significant reduction in phosphorylation was 30-142 (Figure 3A, lanes 5 & 6), suggesting that the primary plk1 phosphosite is within the NH2 terminal 29 amino acids. Plk1 frequently targets serines or threonines in close proximity (32), and within the first 29 amino acids of survivin, serine 20 and threonine 21 are adjacent. In Silico modelling studies have suggested that S20 is an externally exposed residue, while T21 forms a strong hydrogen bond with a neighbouring residue, and is deeply embedded in the BIR domain. Consistent with its structural importance we found that mutating T21 (T21A or T21V) greatly altered the solubility of the protein, presumably due to problems in its folding in vitro. Given the insolubility of T21 mutants we have interpreted in vitro experiments using these mutants cautiously, and have focussed our attention primarily on S20.

To determine whether serine 20 was a plk1 target site, we mutated it to a non-phosphorylatable alanine (S20A) and repeated the plk1 kinase assay. As shown in Figure 3B, while GST-survivin shows abundant incorporation of 32P (lane 1), this was reduced to background levels in the GST-S20A mutant (compare lane 2 with GST control, lane 3), indicating that S20 is phosphorylated by plk1.

As mentioned above, T21 is also a potential plk1 site, and a further site, T97, which, although outside the region we identified by GST-mapping, lies within an appropriate consensus sequence for plk1 (see discussion). Thus we next tested whether substituting these threonines for alanine could also inhibit plk1 phosphorylation of survivin in vitro. As shown in Figure 3C (left panel) the substitution T21A, also reduced 32P incorporation in the presence of plk1, as did the S20A/T21A double mutant, thus T21 too may be targeted by plk1. By contrast, mutating T97 had no effect on plk1’s ability to

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phosphorylate survivin, nor did mutation of a putative CK2 site, T48A (Figure 3C, right panel). Thus we conclude that plk1 can target survivin at S20 and potentially T21, but not T97 in vitro.

To ascertain whether plk1 phosphorylation of survivin could influence binding of the PBD of plk1, we performed a Far Western experiment (Figure 3D). Untagged recombinant survivin was in vitro phosphorylated with recombinant plk1, the two proteins separated by SDS-PAGE and transferred to nitrocellulose. The membrane was then incubated with recombinantly expressed GST-PBD, and probed with anti-GST antibodies. Under these conditions GST-PBD binding to survivin was initially very low, but increased as phosphorylation of survivin increased. Thus binding of the PBD of plk1 can be regulated by phosphorylation and plk1 itself can prime this association. We next tested a panel of other kinases known to phosphorylate survivin, cdk1, aurora-B and CK2 (R.M.A. Barrett, R. Colnaghi, and SPW, unpublished observations). Despite efficient phosphorylation by each kinase as determined by 32P incorporation (Figure 3E, upper panel), GST-PBD only bound to the plk1 phosphorylated form, confirming that plk1 can regulate its own binding to survivin.

**Characterisation of Survivin Phosphorylation Mutants.**

To begin to understand the functional significance of plk1-mediated regulation of survivin, U2OS cell lines stably expressing GFP, survivin-GFP or C-terminally GFP-tagged S20 and T21 mutants were established and their localisation recorded. As shown in Figure 4A, the non-phosphorylatable S20A, concentrated at centromeres from early prometaphase through to metaphase, while the corresponding (putative) phosphomimic, S20D, was diffusely localised during early stages of mitosis, but nevertheless gained access to the centromeres as the chromosomes congressed. After metaphase, both S20A and S20D localised at the midzone, like wild type, although a tighter association with these structures was often apparent for S20D, compared to S20A. During interphase, when survivin is overexpressed it is predominantly cytoplasmic, and this localisation remained unchanged for S20 mutants (data not shown).

In contrast to the S20 mutants, and despite similar expression levels (Figure 4D), neither T21A nor T21D localised to any of the structures that typically recruit survivin (Figure 4B). Instead they were distributed diffusely throughout the cells with some accumulation at the centrosomes. The similarity in localisation between T21A and T21D suggests that T21D probably represents another non-phosphorylatable form, as opposed to a phosphomimic. Despite this aberrant localisation, but consistent with the viability of these stably expressing cell lines, aurora-B and borealin localisation remained unperturbed in the presence of all forms of ectopically expressed survivin, and no gross abnormalities in chromosome arrangements were observed at any specific stage (Supplementary Figure 1, and data not shown).

To ascertain whether the localisation of alanine substituted S20 or T21 was representative of a non-plk1-phosphorylated survivin, we compared their localisation to survivin-GFP in cells treated with BI 2356, a small molecule inhibitor of plk1 (Figure 4C). BI 2356 treated cells arrested in prometaphase and displayed abundant centromeric survivin-GFP, phenocopying the distribution of S20A further indicating that S20 is the principle plk1 target of survivin. We therefore focussed the remainder of this study on the S20 mutated forms.

Next we asked whether mutation of S20 altered plk1-survivin liaisons in vivo. Immunoprecipitation of the ectopic proteins using anti-GFP antibodies from asynchronous cell lines expressing GFP, survivin-GFP, S20A or S20D (Figure 4E), which had been transiently transfected with plk1 kinase, revealed that S20A reduced the plk1-survivin interaction. These data are consistent with the lack of S20A phosphorylation in vitro (Figure 1B) and the ability of plk1 phosphorylation to facilitate binding of the PBD in the Far Western assay (Figure 1E).

**S20A is a Dominant Negative Mutant that cannot Correct Syntelically Attached Chromosomes.**

To determine whether S20 mutants influenced the accuracy and timing of mitosis we turned to live imaging analyses. When released from mitotic arrest using colcemid, and observed at low magnification, no major changes were observed between the different cell lines, although, S20D expressing cells appeared to complete division and re-adhere to the substrate slightly more readily (Supplementary Figure 2, and data not shown). Similarly no clear differences in cell division were visible during
initial observations of asynchronous cells, even at high resolution. However, one of the principle roles of the CPPs is to correct erroneously attached chromosomes during their congression to the metaphase plate, and such defects are often difficult to detect. Thus to discover whether S20 mutants were competent in this role, we exacerbated the presence of misaligned chromosomes by invoking a mitotic arrest using the Eg5 inhibitor dimethylastron, which inhibits centrosome separation, causing formation of a monopolar spindle to which all chromosomes attach in a syntelic fashion. As this treatment is reversible, we assayed the ability of the different cell populations to correct positioning of maloriented chromosomes generated by this treatment, and their subsequent ability to divide. Gratifyingly, this experiment revealed that the non-phosphorylatable version of survivin, S20A, was unable to correct all maloriented chromosomes within the cell, and strikingly, cells expressing S20A were able to proceed into anaphase and cytokinesis regardless of the misaligned chromosomes, and without a prolonged delay (Figure 5B upper panel and 5D), although alignment was achieved in some cases (see Figure 5B, lower panel). In contrast to the malfunctioning S20A expressing cells, both the control survivin and S20D expressing cells successfully corrected all erroneously connected chromosomes (compare Figures 5A and C), with S20D completing the tasks of chromosome alignment and cytokinesis slightly faster than those expressing wild type survivin (Figures 5A, C-G). Note that the time spent waiting anaphase was similar between these two populations (Figure 5F). From these data we conclude that S20A is a dominant negative mutant that cannot correct maloriented chromosomes, while S20D operates more efficiently during prometaphase and cytokinesis than its wild type counterpart. Immunostaining of each line after overnight treatment with dimethylastron demonstrated that, the spindle checkpoint protein, BubR1, was highly abundant on the kinetochores of syntelic chromosomes in populations expressing wild type and S20D survivin, but reduced, although not completely absent in cells expressing S20A (Figure 5H).

Finally, to demonstrate interaction between survivin and plk1 during correction of syntelic chromosomes, we immunoprecipitated endogenous plk1 from extracts prepared at 30 minute intervals post-release from dimethylastron treatment. Immunoblotting of whole cell extracts indicated that endogenous plk1 expression declines as cells exit mitosis, concomitant with the reduction in phosphorylated H3 levels (Figure 5I, lower panel). Accordingly, when immunoprecipitated, endogenous plk1 was detectable at 0 and 30 minutes post-release, at which times endogenous survivin co-immunoprecipitated with it (Figure 5I, upper panel). Thus, while our overexpression data in Figure 1 demonstrated we can detect interactions between plk1 and survivin when they are in sufficient quantity, when monitoring interaction of the endogenous forms, the greater abundance of plk1 during early mitotic events makes their liaisons are more readily detected during chromosome congression.

**Survivin$_{S20A}$ cannot support cell proliferation.** Next we used siRNA to deplete endogenous survivin from cells expressing survivin-GFP or siRNA-resistant S20 mutants and assessed their ability to support cell division. Initially upon removal of the endogenous protein (48h) both S20 mutants localised normally during mitosis with S20D gaining access to the centromeres more efficiently in its absence (data not shown). Depletion of survivin from the control culture expressing siRNA sensitive survivin-GFP inhibited cell proliferation with no increase in cell number apparent even at the earliest time point (Figure 6A, left panel). FACS profiling of this population at 48h indicated a loss of G1 cells with 2N content, an accumulation of cells with >4N, and a concomitant increase in cells with sub-2N DNA, indicative of cells undergoing apoptosis (Figure 6B, left panel). By 96h few cells remained in the control population and those that did were highly multinucleated, as judged by fluorescence imaging of F-actin and DNA (Figure 6C, left panel). By contrast, although their growth rates were reduced, populations expressing S20A and S20D continued to proliferate up to 72h post-RNAi (Figure 6A, centre and right panels). FACS profiling at 48h indicated an accumulation of cells with 4N content in both populations but negligible sub-2N or >4N cells (Figure 6B, centre and right panels). Interestingly, although S20A sustained growth and viability for 72h post-RNAi, the persistent absence of the endogenous form eventually inhibited these cells from proliferating, while S20D cells continued to grow, as indicated at the 96h time point. To confirm the differences in their proliferative capacity, we assessed the clonogenic potential of
cells from each population 96h post-RNAi by replating them at low density and counting colony formation 7 days later. In accordance with the proliferation data cells expressing S20A were unable to form viable colonies, but those expressing S20D formed colonies of >50 cells (data not shown). Despite cessation of proliferation DNA-FACS profiling indicated that the cell cycle distribution of the S20A population at 48 and 96h was comparable to the distribution of the S20D population, which was still growing. Finally we used fluorescence imaging to assess the phenotype of these cells at 96h. As expected, any remaining cells in the control population were polyplloid (Figure 6C, left panel), while the S20A and S20D populations had nuclei of normal size (Figure 6C, right panels). Strikingly, however, the nuclei in the S20A population showed a strong accumulation of S20A in foci, (Figure 6C, middle). Immunoprobing with anti-CENPC antibodies at high resolution, revealed that some of these foci were sites of centromere clustering (Figure 6C, lower panel).

**S20 survivin mutants can inhibit apoptosis.**

In addition to their essential functions in mitosis, survivin and plk1 kinase are also implicated in cell death pathways. To test whether mutation of the plk1 relevant phosphosite of survivin affected its ability to inhibit apoptosis, U2OS cells expressing GFP, wild type survivin, S20A or S20D were treated with TRAIL at 30 minute intervals and analysed using a tetrapeptide cleavage assay for caspase-3 activity. As shown in Figure 7, wild type survivin, S20A and S20D expression all protected cells from apoptosis. These data suggest that the phosphorylation status of S20 is not important for survivin’s anti-apoptotic function within the extrinsic apoptosis pathway.

**Discussion.**

Survivin and plk1 kinase are both cancer-relevant proteins that are involved in cell division and cell death. Given the similarity in their expression, localisation, and the response of cells to their ablation, we hypothesised that they may act in concert during these events to fulfil their duties. Here we show that survivin and plk1 do indeed interact in vivo as recently reported by Feng and co-workers (31), and extend these observations to demonstrate that their interaction can occur directly. Importantly, we show for the first time that survivin is a plk1 substrate and identify S20 as the principle residue targeted by plk1 and that phosphorylation at this site is required for cell division and to correct malorientated chromosomes during congression. Finally we demonstrate that although S20-phosphorylation is essential for cell proliferation, it does not affect the ability of survivin to inhibit apoptosis.

**Plk1 phosphorylates survivin principally on S20.**

At the outset of this study we were excited to discover that within survivin’s highly conserved central region, residues 95-100 (ELTΨLGE), follow precisely the canonical plk1 phosphorylation consensus: D/E-X-S/T-Ψ-X-D/E (where X is any residue, S/T is the phospho-target, and Ψ is hydrophobic; (33)). This region is engaged in a multitude of survivin activities including its homodimerisation (34,35), its interaction with its mitotic partner, borealin (36,37), and is also an integral part of its nuclear exportation signal (38,39). However, despite the apparently perfect consensus, its ideal positioning to act as a phosphor-switch, and the precedence that nuclear-cytoplasmic shuttling of cyclin B1 (40) and MKLP1 are regulated by plk1 (27,41), in vitro phosphorylation of survivin by plk1 was unaffected when threonine 97 was substituted for a non-phosphorylatable alanine (T97A). Instead, GST-phosphor-mapping and site-directed mutagenesis revealed S20 as the principle plk1-target site, a site previously shown to be regulated by cAMP protein kinase A, PKA (42). We also noted that the residue neighbouring S20, T21, may be targeted by plk1, but due to its structural importance within the molecule we have interpreted these data with caution. Moreover, despite considerable effort, phosphopeptide analysis of this region has so far evaded detection by Mass Spectrometry. Treatment with BI 2356, however, revealed that cells arrested in prometaphase in response to the plk1 inhibition, have abundant survivin-GFP at their centromeres, a pattern closely phenocopying S20A localisation, and distinct from the distribution of T21A (this study) and T97A (38), lending further credence to our conclusion that S20 is the principle plk1 target of survivin.

**Survivin is a late docking partner of plk1.**

In addition to its phosphorylation consensus, binding of the PBD of plk1 can be modulated both positively and negatively by prior phosphorylation of its substrate (32). For
example cdk1 does both: it primes plk1 binding and phosphorylation of early mitotic partners such as cyclin B1 and cdc25 thereby promoting mitotic entry (40,43), while simultaneously inhibiting binding and phosphorylation of anaphase relevant plk1-substrates, including the microtubule binding proteins MKLP2 and PRC1 (28,29). Docking and phosphorylation of these late mitotic-plk1 partners is mediated by kinases distinct from cdk1, and can be regulated by plk1 itself (28,29). Our data show that plk1 phosphorylates survivin at S20 and that although binding can occur independently of phosphorylation, binding of the PBD can be enhanced by plk1 phosphorylation, but not cdk1, aurora-B or CK2, suggesting that it is a docking partner of plk1 during, but not exclusively, in late mitosis. Consistent we this, our expression studies indicate that plk1 and survivin can associate throughout mitosis. Survivin is not the only CPP regulated by plk1, indeed Goto et al., (2006), reported that cdk1 phosphorylates INCENP at T388 and that phosphorylation at this site is required for plk1 recruitment to the kinetochores, and anaphase onset (44), suggesting that it is also a plk1-docking partner. It will be interesting to discover whether there is a sequential pattern of CPP phosphorylation during mitosis, and whether distinct subcomplexes are regulated by these events. Moreover, as PKA also targets S20 (42), presumably it too can facilitate PBD binding, raising the questions as to whether there is cooperation between plk1 and PKA in the regulation of survivin during the cell cycle.

**Phosphorylation of survivin by plk1 is required for spindle checkpoint response and cell division.**

The most striking observation from our overexpression studies is that S20A, which cannot be phosphorylated by plk1, proceeds through anaphase and cytokinesis despite the presence of maloriented chromosomes. Moreover, in addition to being unable to correct these erroneously attached chromosomes, cells expressing S20A do not delay entrance into anaphase and divide within the normal schedule experienced by cells expressing wild type survivin. This dominant negative phenotype is reminiscent of the response of survivin depleted cells to monastrol and taxol, conditions that satisfy the checkpoint protein Mad2, forcing the cells to rely completely on the tension sensor BubR1, to detect any errors (25,26). Using RNAi, in these studies we, and others, demonstrated that the persistence of BubR1 at centromeres lacking tension is dependent upon the presence of survivin. Further work revealed that while survivin is phosphorylated by aurora-B during prometaphase, to achieve chromosome alignment, the aurora-B target of survivin (T117), must be dephosphorylated (7). The current data extend these observations confirming that the presence of survivin at the centromere alone is insufficient to sustain the spindle tension checkpoint, as indicated by BubR1 immunolocalisation, and demonstrating for the first time that survivin must be phosphorylated at S20 by plk1 kinase for accurate congression and safe passage into anaphase. Furthermore, data obtained using our (putative) phosphomimic, S20D, suggests that plk1-phosphorylated survivin corrects chromosome alignment and executes cytokinesis more efficiently than the wild type version.

Using RNAi complementation we observed that expression of S20A alone inhibited cell proliferation, while S20D supported cell growth, further demonstrating that phosphorylation of survivin at S20 is critical for cell division. Cell cycle analysis by FACS profiling and phenotypic inspection of the S20A mutant showed that there was an accumulation of cells with 4N content, from 48h, and that a slight increase in cell death was occurring at 96h. At this late time point, S20A was abnormally present and abundant in nuclear foci. These observations suggest that phosphorylation of survivin by plk1 at the metaphase-anaphase transition may facilitate its transfer from the centromeres to the anaphase spindle, which could result from either an increased affinity for microtubules, or alternatively decreased affinity for the centromere. However, at least one report has suggested that the transfer of the CPPs from the centromere to the cleavage furrow can occur in the absence of plk1 (22).

**S20 phosphorylation is dispensable for survivin’s IAP activity.**

S20 was previously identified as a PKA target (42). These authors further reported that the mutation S20A augmented survivin’s IAP activity and attributed the enhanced cytoprotection conferred by its expression, to an increased affinity for XIAP, an IAP family member with which survivin has been shown to cooperate to inhibit apoptosis (42,45). In agreement with this report we find that S20A is
able to inhibit TRAIL-mediated apoptosis, but find that the phosphomimic is also protective. Thus we conclude that phosphorylation at this site, which can be achieved by plk1 or PKA, does not play a primary role in the regulation of survivin’s IAP activity.

In summary our data presented herein are the first to demonstrate that survivin is a plk1 substrate and have identified S20 as the major plk1-phosphorylation site. Using paired S20 mutants, we have shown that phosphorylation at S20 is required for survivin chromosome alignment and appropriate response to the spindle tension checkpoint. We also provided data indicating that phosphoregulation of S20 by plk1 provides a new route to separate the mitotic and anti-apoptotic functions of survivin. Intriguingly we recently reported that survivin’s “dual” roles could be bifurcated by altering the status of its cdk1 site, T34 (9), and it is of note that many mitotic proteins are regulated by both cdk1 and plk1. Clearly phosphoregulation of survivin alone, and as an integral member of the CPC, is not one-dimensional, but part of a highly complex phosphorylation network, posing considerable challenge to unravelling the molecular consequences of these modifications both in mitosis and apoptosis.
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Figure Legends.

Figure 1: Colocalisation and interaction between survivin and plk1 during mitosis. (A) Immunolocalisation of plk1 kinase (Ab14209; red) in formaldehyde fixed U2OS cells expressing survivin-GFP (green), counterstained with DAPI to visualise the chromosomes (blue). A subpopulation of plk1 colocalises (as indicated in yellow) with survivin at the centromeres during prometaphase and metaphase. At anaphase and telophase all plk1 transfers to the central spindle and midbody where it colocalises with survivin. Bar 5µm. (B) Reciprocal immunoprecipitation (IP) of GFP or survivin-GFP using anti-GFP antibodies, and plk1 kinase using anti-plk1 antibodies, in U2OS cells transiently transfected with plasmids encoding GFP and plk1, or survivin-GFP and plk1 as indicated. Survivin-GFP co-immunoprecipitated with plk1, and conversely, plk1 co-immunoprecipitated with survivin-GFP, but not with GFP alone. IB= immunoblot. (C & D) Immunoprecipitation using anti-plk1 kinase antibodies was repeated on synchronised cell extracts prepared from survivin-GFP expressing U2OS cells (C), or U2OS cells with no ectopic survivin (D). Cells were arrested in mitosis using a sequential thymidine-nocodazole regime (time 0) and released for 30, 60 or 90 minutes as indicated. Accompanying whole cell extracts (WCE) were probed with anti-cyclinB1 antibodies to indicate release from mitosis, and anti-actin included as a loading control for the WCEs. Both survivin-GFP (C) and endogenous survivin (D) showed greatest association with ectopic plk1 kinase when cyclin B1 levels were at their lowest, indicating increased affinity between the proteins as cells exit mitosis.

Figure 2: Survivin and plk1 interact directly in vitro. In vitro pulldown assays. GST, or GST-tagged versions of survivin (A), or GST-tagged versions of plk1 (B), were incubated with in vitro translated (IVT) $^{35}$S-methionine labelled plk1 (A), survivin or the C-terminally truncated form of survivin, 1-90 (B). Plk1 interacted directly with GST-survivin and survivin truncations lacking the C-terminal alpha helix. Conversely survivin interacted with full length plk1, its kinase domain (KinD) and its polo-binding domain (PBD), which encompasses two polo boxes (PB1 & PB2), and survivin 1-90 showed strongest interaction with GST-PBD. Note the reduced expression of full length plk1, probably due to decreased stability as indicated by the asterisk. Stick models of survivin and plk1 are shown to illustrate the relevant domains (not drawn to scale).

Figure 3: Survivin is a plk1 substrate. (A-C) In vitro kinase assays showing autoradiograms in the upper panels and corresponding Coomassie Blue (CB) staining of each gel in the lower panels. (A) Recombinant GST, GST-survivin or GST-survivin lacking the first 29 amino acids (30-142), was incubated in vitro with recombinant plk1 in the presence of $^{32}$P-γ ATP. Plk1 phosphorylated GST-survivin (lanes 3 & 4; SVN), but not the GST control (lanes 1 & 2). $^{32}$P incorporation was greatly reduced in the N-terminal truncation, GST-30-142 (lanes 5 & 6) suggesting that plk1 phosphorylation occurs within the first 30 amino acids. We also noted that plk1 phosphorylated itself (Plk1$^{\text{IVT}}$). (B) In vitro plk1-kinase assay to investigate S20. Plk1 phosphorylated wild type survivin (GST-SVN, lane 1), but not GST-S20A (lane 2) or GST (lane 3). (C) The plk1-kinase assay was repeated using the point mutants indicated. Note that although T21 and S20AT21A mutants showed reduced phosphorylation, they were only partially soluble and these preparations required considerably more extract/beads than other samples. T48A and T97A mutants were phosphorylated as wild type. (D) Far Western. Untagged recombinant survivin (GST-tag removed by thrombin cleavage) was phosphorylated in vitro with plk1, as described above. The reaction was denatured, survivin and plk1 separated by electrophoresis, and transferred to nitrocellulose. The membrane was then incubated with GST-PBD, and probed with anti-GST antibodies. Upper panel: autorad showing survivin phosphorylation ($^{32}$P-incorporation) from 10-40 minutes (lanes 1-3), and the ability of GW843682X to inhibit phosphorylation (lane 4). Lane 5 is included as a negative control. Middle panel: Coomassie stained gel (CB) to demonstrate equality in loading. Lower panel: Far Western (FW) showing increasing association between denatured survivin and GST-PBD with increasing phosphorylation (lanes 1-3). This interaction is abolished in the presence of the plk1 inhibitor GW843682X (lane 4). (E) Far Western using survivin pre-phosphorylated with cdk1, aurora-B, Plk1 or CK2, as indicated. Only Plk1 phosphorylation promoted GST-PBD binding under these conditions.
Figure 4: Analysis of S20 and T21 survivin mutants. U2OS lines stably expressing survivin-GFP, or survivin-GFP bearing the mutations S20A, S20D, T21A, T21D as indicated, were examined during mitosis using fluorescence microscopy. In (A) anti-tubulin was used to immunoprobe cells for microtubules (red). Tubulin localisation was omitted in (B) to aid clarity of GFP signal, which was more diffuse. In all panels chromosomes are stained with DAPI (blue). S20A was clearly localised at the centromeres from prophase through to metaphase, while this localisation was weaker and more variable for S20D. Interestingly the converse was the case during anaphase and telophase with S20D showing more complete localisation to the midzone and midbody than S20A. (B) Neither T21A nor T21D displayed localisation typical of survivin-GFP, instead these forms were diffusely distributed throughout the cell, with some concentration at the centrosomes. (C) Cells expressing wild type survivin were incubated in the absence (survivin) or presence of BI 2356 (survivin+BI), and survivin-GFP localisation compared with the distribution of survivin bearing alanine substitutions at S20 or T21, as indicated. BI 2356 treatment induced a prometaphase arrest and did not affect survivin-GFP accumulation at the centromeres. S20A phenocopied BI treatment, while T21A localisation was distinct. Bars 5μm. (D) Immunoblot with anti-GFP antibodies and anti-tubulin as a loading control to compare levels of expression in each cell line. (E) Immunoprecipitation (IP) using anti-GFP antibodies in the cell lines indicated after transient expression of plk1 kinase. WCE: whole cell extracts probed with anti-plk1 and anti-survivin antibodies revealed that transient plk1 expression was similar in each line, and survivin variants were also present at similar levels. Anti-GFP antibodies were used to immunoprecipitate GFP, or the survivin-GFP variant of interest, and immunoblots probed with anti-plk1 antibodies. S20A exhibited reduced binding to plk1 compared with wild type survivin, or S20D under these conditions.

Figure 5: S20A cannot correct synthetically oriented chromosomes. U2OS lines stably expressing survivin-GFP (A;wt), or S20A-GFP (B;S20A) or S20D-GFP (C;S20D), as indicated, were treated overnight with 2 μM dimethylastron and harvested by mitotic shake off, before extensive washing in PBS and release into prewarmed drug-free CO₂-independent medium. A Z-sweep of 40 images (0.3 μm) was recorded for each sample at multiple positions in DIC (upper images in each set) and GFP (lower image) at 2 minute intervals, using a x100 (NA 1.4) oil immersion lens. Gallery images are 2D projections of z-stacks of every fifth time point in each sequence (i.e. 10 minutes apart, as indicated numerically top right). S20A cells entered anaphase despite the persistence of misaligned chromosomes (arrows), while S20D cells divided more rapidly. (D-G) Timing of specific mitotic events. As S20A cells rarely achieved chromosome alignment, only the total duration of division could be assessed (D), while the time taken to congress chromosomes to the metaphase plate (E), the duration of time spent at the metaphase plate (F) and time taken from achieving alignment to midbody formation is given for wild type and S20D lines. (G) Wide field imaging of cells treated for 16 h with dimethylastron, and immunoprobed with anti-BubR1 antibodies (red) revealed diminished BubR1 signal at kinetochores of S20A cells. (I, upper panel) Immunoprecipitation of endogenous plk1 using anti-plk1 antibodies, revealed detectable co-immunoprecipitation of endogenous survivin at 0 and 30 minutes post-release from dimethylastron. Whole cell extracts (WCE) were probed with anti-phosphoH3 antibodies to confirm transition through mitosis (lower panel). Bars 5μm.

Figure 6: S20D, but not S20A, supports cell proliferation. U2OS cells expressing survivin-GFP, sensitive (ie. Not resistant, NR) to siRNA, or siRNA resistant (R) S20A-GFP, or S20D-GFP were subjected to siRNA. Data presented are from a representative experiment, performed three independent times. (A) Cell proliferation was monitored for 96h in control or survivin specific siRNA exposed cultures. In the control population expressing siRNA sensitive survivin (NR)-GFP, survivin specific siRNA prevented cell proliferation from 48h. (B) FACS profiling of DNA content 48h (upper) and 96h (lower) post depletion of endogenous survivin. In contrast, neither S20A nor S20D cells became polyploid at 48h or 96h. (C) Cells were examined microscopically at 96h after staining with rhodamine-phalloidin, and DAPI, to visualise F-actin (red) and DNA (blue) respectively. All GFP signal (green) was eliminated in cells expressing siRNA sensitive survivin-GFP, and any remaining cells were multinucleated. Cells expressing S20A-GFP were mononucleated and S20A-GFP was present in bright nuclear foci. S20D-GFP cells were also mononucleated but S20D-GFP was mostly cytoplasmic. High resolution imaging (x 100) of S20A cells immunoprobed with anti-CENPC.
antibodies 96h post-survivin siRNA revealed that some of these nuclear foci were sites of centromere clustering (arrows). Bars 5µm.

**Figure 7: Survivin’s IAP activity is retained in both S20 mutants.** (A) A fluorogenic (Ac-DEVD-AMC) caspase activity assay was performed on extracts prepared from U2OS cells expressing GFP, survivin-GFP, S20A-GFP or S20D-GFP, which had been exposed to TRAIL for 0, 30, 60 or 90 minutes. Immunoblotting with anti-GFP antibodies, and anti-tubulin as a loading control, revealed that all versions were expressed at similar levels (B) and were able to suppress caspase-3 activity. RFU- relative fluorescence units.
Table 1: Mapping Plk1 phosphorylation site(s) of survivin.

| Survivin construct | Phosphorylation | Expression | Solubility  |
|--------------------|-----------------|------------|-------------|
| 1-142 (full length) | ++              | Good       | Soluble     |
| 1-115              | ++              | Good       | Soluble     |
| 1-90               | ++              | Good       | Soluble     |
| 90-142             | -               | Good       | Soluble     |
| 98-142             | -               | Good       | Soluble     |
| 30-142             | -               | Good       | Soluble     |
| S20A               | -               | Good       | Soluble     |
| T21A               | -               | Good       | Mostly insoluble |
| S20AT21A           | -               | Good       | Mostly insoluble |
| T48A               | ++              | Good       | Soluble     |
| T97A               | ++              | Good       | Soluble     |

Abbreviations.
BIR: baculovirus inhibitor of apoptosis repeat; CPC: chromosomal passenger complex; CPP: chromosomal passenger protein; IAP: inhibitor of apoptosis protein; INCENP: inner centromeric protein; PBD: polo binding domain; Plk1: polo-like kinase 1; TRAIL: tumour necrosis factor receptor apoptosis inducing ligand.

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Figure 1
Colnaghi & Wheatley.
Figure 2
Colnaghi & Wheatley.
Figure 3
Colnaghi & Wheatley.
Figure 4
Collaghi & Wheatley.
Figure 4 continued, Colnaghi & Wheatley.
Figure 5
Colnaghi & Wheatley.
Figure 5 (continued)
Colnaghi & Wheatley.
Figure 6
Colnaghi & Wheatley.
Figure 7
Colnaghi & Wheatley.
