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Report

Phosphorylation of survivin at threonine 34 inhibits its mitotic function and enhances its cytoprotective activity

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Abbreviations: IAP, inhibitor of apoptosis; cdk1, cyclin dependent kinase-1; CPP, chromosome passenger protein; GFP, green fluorescent protein; TRAIL, TNF-related apoptosis inducing ligand; WT, wild type

Key words: apoptosis, mitosis, survivin

Survivin is an essential chromosomal passenger protein required for mitotic progression. It is also an inhibitor of apoptosis and can prevent caspase-mediated cell death. In addition, survivin levels are elevated in cancer cells where its presence correlates with increased resistance to chemo- and radio-therapy, which makes it an attractive target for novel anti-cancer strategies. Interestingly, survivin is phosphorylated by the mitotic kinase, cdk1, and a non-phosphorylatable form, survivin_{T34A}, cannot inhibit apoptosis. Here we rigorously test the ability of survivin_{T34A} and its corresponding phosphomimetic, survivin_{T34E}, to promote cell viability through survivin's dual roles. The effects of these mutations are diametrically opposed: survivin_{T34A} accelerates cell proliferation and promotes apoptosis, whereas survivin_{T34E} retards growth and promotes survival. Thus the phosphorylation status of survivin at T34 is pivotal to a cell's decision to live or die.

Introduction

In normal cells survivin is expressed during G₂/M and is essential for mitosis and cytokinesis. When overexpressed in tumors, however, it is present throughout the cell cycle. Survivin expression confers resistance to irradiation and chemotherapy,¹⁻³ and correlates with refractory tumors in the clinic.^{4,5}

In addition to its role in mitosis, survivin is a member of the inhibitor of apoptosis family, IAP.⁶ Although survivin undoubtedly suppresses caspase activity, how this is achieved is unclear with evidence for⁷ and against,⁸ direct caspase interaction. Survivin immunoprecipitates with cdk1 during mitosis,⁹ and is phosphorylated by it at threonine 34.¹⁰ T34 lies within the IAP-specific baculovirus inhibitor of apoptosis repeat domain and mutation of T34 to a non-phosphorylatable alanine abolishes survivin's cytoprotective activity

and decreases its stability.¹⁰⁻¹² Consistent with this, suppression of T34 phosphorylation by cdk1 inhibition also enhances apoptosis.¹² Intriguingly, survivin is the only IAP phosphorylated by cdk1. Thus, together with its mitotic function, the hypothesis that survivin-cdk1 plays a central role in determining whether a cell should live or die at this critical juncture is attractive, see O'Connor et al. (2000).

While it is known that survivin_{T34A} supports growth but cannot inhibit apoptosis,¹³ the functional repertoire of the corresponding phosphomimetic, survivin_{T34E} has not been characterized. Here we examine whether these mutants can support life and inhibit death in cultured human cells. Our data show that modification of T34 both negatively and positively impacts on survivin function and is pivotal in balancing life and death at the cellular level.

Results and Discussion

Survivin T34 mutants have opposing effects on cell proliferation rates. Proliferation of HeLa cells stably expressing GFP, survivin-GFP, survivin_{T34A}-GFP or survivin_{T34E}-GFP was monitored at 24 h intervals over 4 days (Fig. 1A). Cells expressing GFP alone and survivin-GFP (WT) grew with similar kinetics. In contrast, cells expressing survivin_{T34A}-GFP (T34A) grew two fold faster than controls, and cells expressing survivin_{T34E}-GFP (T34E) grew 2.5 fold slower.

Survivin T34 mutants localize normally during mitosis. We next asked whether these versions localize correctly during mitosis. As for survivin-GFP both mutant forms localized to the centromeres (prometaphase-metaphase), midzone microtubules (anaphase) and midbody (cytokinesis) when endogenous survivin was present (Fig. 1B). However, in addition, survivin_{T34A}-GFP appeared to track along the anaphase spindle, rather than being confined to the region of microtubule overlap, and was sometimes present at the centrosome. Our T34A data are in agreement with refs.^{13,14} The localization of survivin_{T34E}-GFP is consistent with the localization of T34-phosphorylated as described by O'Connor et al. (2000), using phosphospecific antibodies, lending credence to survivin_{T34E} as a phosphomimetic.

T34A, but not T34E, supports growth after depletion of endogenous survivin. To ascertain whether these forms of survivin could

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Figure 1. Cell cycle characteristics of stable cell lines. (A) Proliferation of HeLa cells stably expressing the constructs indicated. The mutation T34A accelerated growth, while T34E reduced it. (B) Cells expressing the versions of survivin-GFP (green) indicated were fixed and immunoprobed to reveal microtubules (red), and chromosomes (blue). These mutants localised normally although survivin_{T34A}-GFP accumulated additionally at the centrosomes and along the anaphase microtubules (arrow). Bars 5 μ m.

support cell proliferation in the absence of endogenous survivin, asynchronous cultures were treated with control or survivin specific siRNA, sec.¹⁵ Survivin_{T34A}-GFP, but not survivin_{T34E}-GFP, supported cell proliferation after survivin depletion (Fig. 2A; depletion was confirmed by immunoblotting, data not shown). Next the number of mitotic cells was monitored by immunostaining with anti-phospho-H3^{ser10} antibodies. Consistent with the growth curves no change in the mitotic index was observed in survivin_{T34A}-GFP cells. However, mitotic cells were rarely seen in the survivin_{T34E}-GFP population even without survivin depletion (Fig. 2B). In addition, the incidence of multinucleation revealed that only survivin_{T34A}-GFP substituted for loss of endogenous survivin as assessed by immunofluorescence (Fig. 2C), and confirmed by FACS analysis (Fig. 2D). Note that DNA-FACS profiling does not distinguish between binucleated and G₂/M cells, thus although there are fewer mitotic survivin_{T34E}-GFP cells, as more are multinucleated (or in G₂), the control survivin_{T34E}-GFP population has an apparently normal distribution. Importantly, upon survivin depletion the FACS profile remained normal for survivin_{T34A}-GFP cells, but the number of 4N cells significantly increased in the survivin_{T34E}-GFP population, suggesting that cell division failed.

To determine the fate of cells expressing only the ectopic form of survivin cells were imaged for 48 h from 24 h post-RNAi (Fig. 3A and B). While 96% of survivin-GFP and 95% of survivin_{T34A}-GFP cells executed mitosis normally, cells expressing survivin_{T34E}-GFP had a number of fates: 6.5% completed mitosis, 14.6% persisted in mitosis for the duration of filming and 15.5% exited mitosis inappropriately yet lived. The majority (46.3%) failed mitosis and died, and the remainder (17.1%) exited mitosis, but failed to divide. Thus consistent with Lens et al. (2006), survivin_{T34A}-GFP can restore survivin's mitotic function. However, our data extends this observation demonstrating that the corresponding phosphomimetic is mitotically incompetent, thus we conclude that phosphorylation at T34 inhibits mitosis.

Localization of CPPs and BubR1 in T34E cells. For successful cell division the complete chromosomal passenger complex is required. We next examined the localization of the CPPs aurora-B and borealin during mitosis in cells expressing only the ectopic forms. Like survivin-GFP (Fig. 3C),

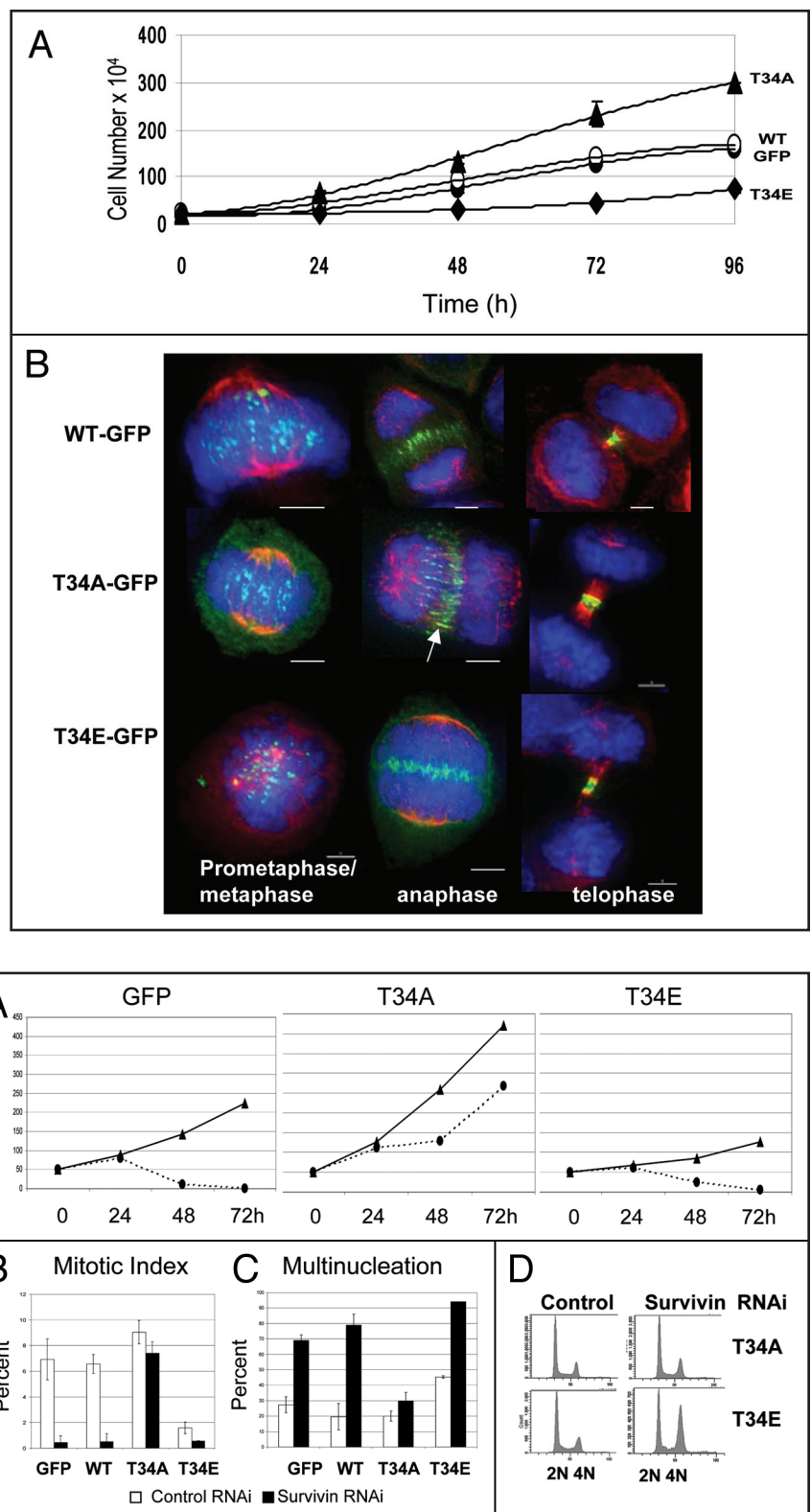


Figure 2. Repertoire of T34 mutants in the absence of endogenous survivin. Endogenous survivin was depleted by siRNA in the lines indicated. (A) Cell proliferation was assessed over 72 h after incubation with control (triangles) or survivin specific (circles) siRNA. Clearly survivin_{T34A}-GFP supported cell growth while survivin_{T34E}-GFP did not. (B) Mitotic staging was assessed by immunofluorescence imaging using anti-phospho-H3 antibodies. (C) Multinucleation was assessed by immunostaining for microtubules and DNA. (D) For FACS analysis cells were stained 48 h post-RNAi with propidium iodide to assess DNA content. After survivin depletion the number of 4N cells increased in the survivin_{T34E}-GFP population, while survivin_{T34A}-GFP cells were unaffected.

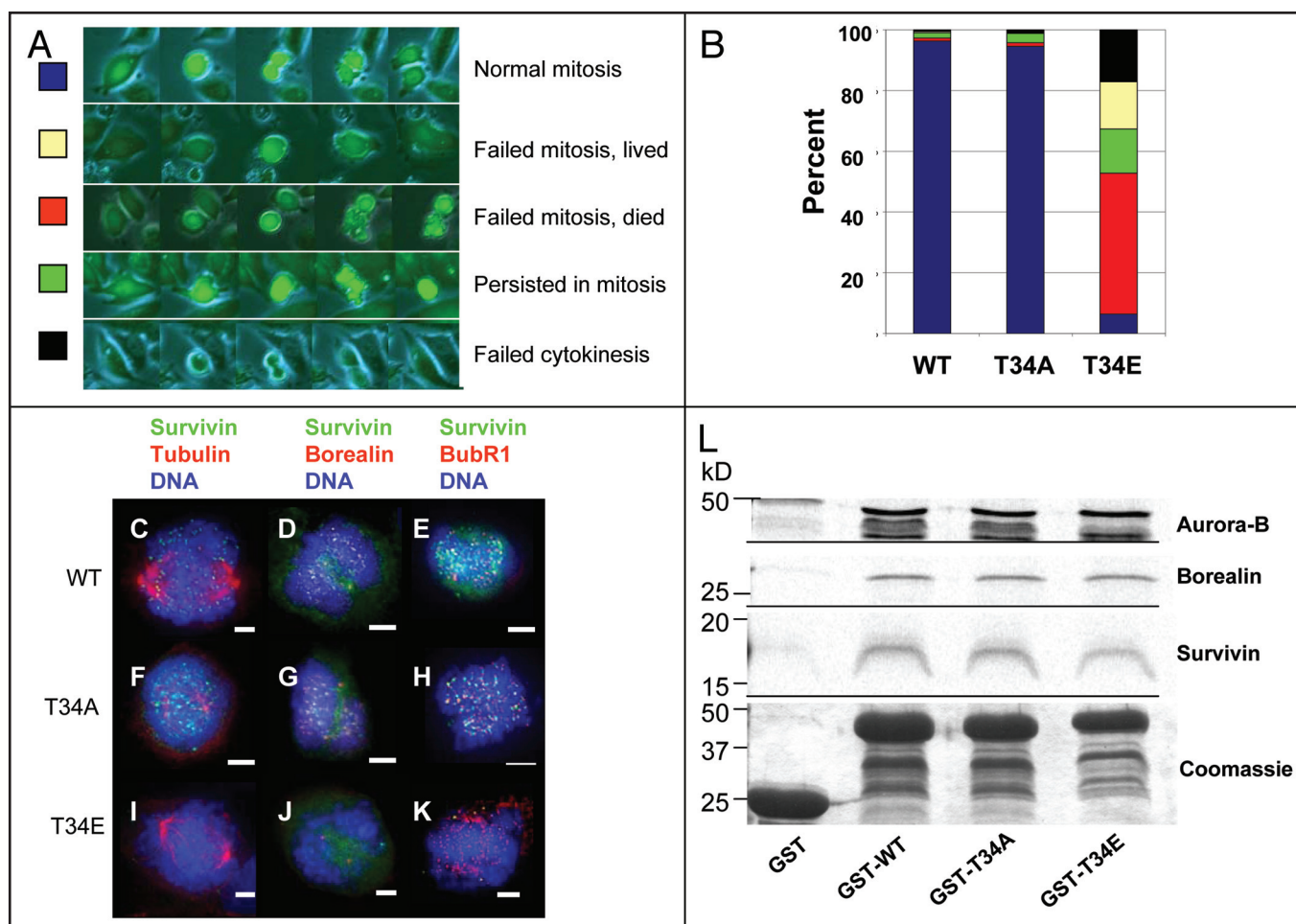


Figure 3. T34E inhibits mitosis. Cells were followed by time-lapse microscopy. Survivin-GFP (96%) and survivin_{T34A}-GFP (95%) expressing cells completed mitosis normally, while survivin_{T34E}-GFP had a number of different fates, as depicted in (A) and quantified in (B): the majority (46.3%) failed to align their chromosomes then died (red); 14.6% persisted in mitosis (green); 15.5% failed to align their chromosomes then exited mitosis but lived (yellow); 6.5% divided normally (blue); and 17.1% exited mitosis but failed cytokinesis (black). (C–K) Immunolocalisation of survivin, borealin and BubR1 48 h post-survivin depletion. Survivin_{T34A}-GFP localised normally when expressed alone, and localisation of borealin and BubR1 was unimpaired. However, neither survivin_{T34E}-GFP, nor borealin was centromeric, although BubR1 was still present. Bars 5 μ m. (L) GST-pull down assay to assess whether binding to *in vitro* translated CPPs. As shown in the autorad (³⁵S-methionine), all versions bound wild type survivin, aurora-B and borealin.

survivin_{T34A}-GFP localized to the centromeres in prometaphase after survivin RNAi (Fig. 3F), and efficiently recruited borealin and aurora-B (Fig. 3G, and data not shown). In stark contrast, in the rare mitotic survivin_{T34E}-GFP cells observed, chromosomes were scattered in pseudoprometaphase and survivin_{T34E}-GFP was unable to bind to centromeres (Fig. 3I), and neither aurora-B nor borealin was recruited (Fig. 3J, and data not shown). To ascertain whether centromeric binding of survivin_{T34E}-GFP failed due to altered affinity for its mitotic partners, we performed GST-pull downs with CPPs (Fig. 3L). Consistent with the interdependence of their localization both survivin_{T34A}-GFP and survivin_{T34E}-GFP were able to bind aurora-B, borealin, and survivin, indicating that mutation of T34 does not affect the integrity of the CPP complex.

Depletion of survivin compromises the spindle checkpoint by destabilising BubR1 at the kinetochores.^{15,16} Hence we observed BubR1 in these lines after depletion of endogenous survivin. BubR1 bound to the kinetochores in the presence of either survivin_{T34A}-GFP or survivin_{T34E}-GFP indicating that the tension-spindle checkpoint is intact in each case (Fig. 3E, H and K). Our data are consistent

with Lens et al. (2006), who showed that survivin_{T34A}-GFP was mitotically competent, able to recruit borealin, aurora-B and BubR1 to the centromeres/kinetochores, and could elicit the spindle checkpoint in response to paclitaxel. Lens et al. (2006) also suggested that survivin_{T34A}-GFP could act as a dominant negative, although in their hands the cell cycle was unaffected by its overexpression.¹³ While we concur that the cell cycle distribution remains the same in these cells, Figure 1 suggests that survivin_{T34A}-GFP expedites the cell cycle.

T34E enhances survivin's cytoprotective activity. Having established that T34 mutations affect the rate of cell proliferation, we asked whether these versions affected survivin's anti-apoptotic activities. Overexpression of survivin-GFP protects cells against apoptosis induced by TRAIL,^{2,3} and the fact that T34A abrogates this function is established.¹⁰ Here asynchronous cells were treated with TRAIL for 15, 30, 45 or 60 minutes: note that endogenous survivin was present in these experiments. As expected, survivin overexpression protected cells against apoptosis (Fig. 4A). Although survivin_{T34A}-GFP can induce spontaneous apoptosis in cancer cells,^{17,18} we

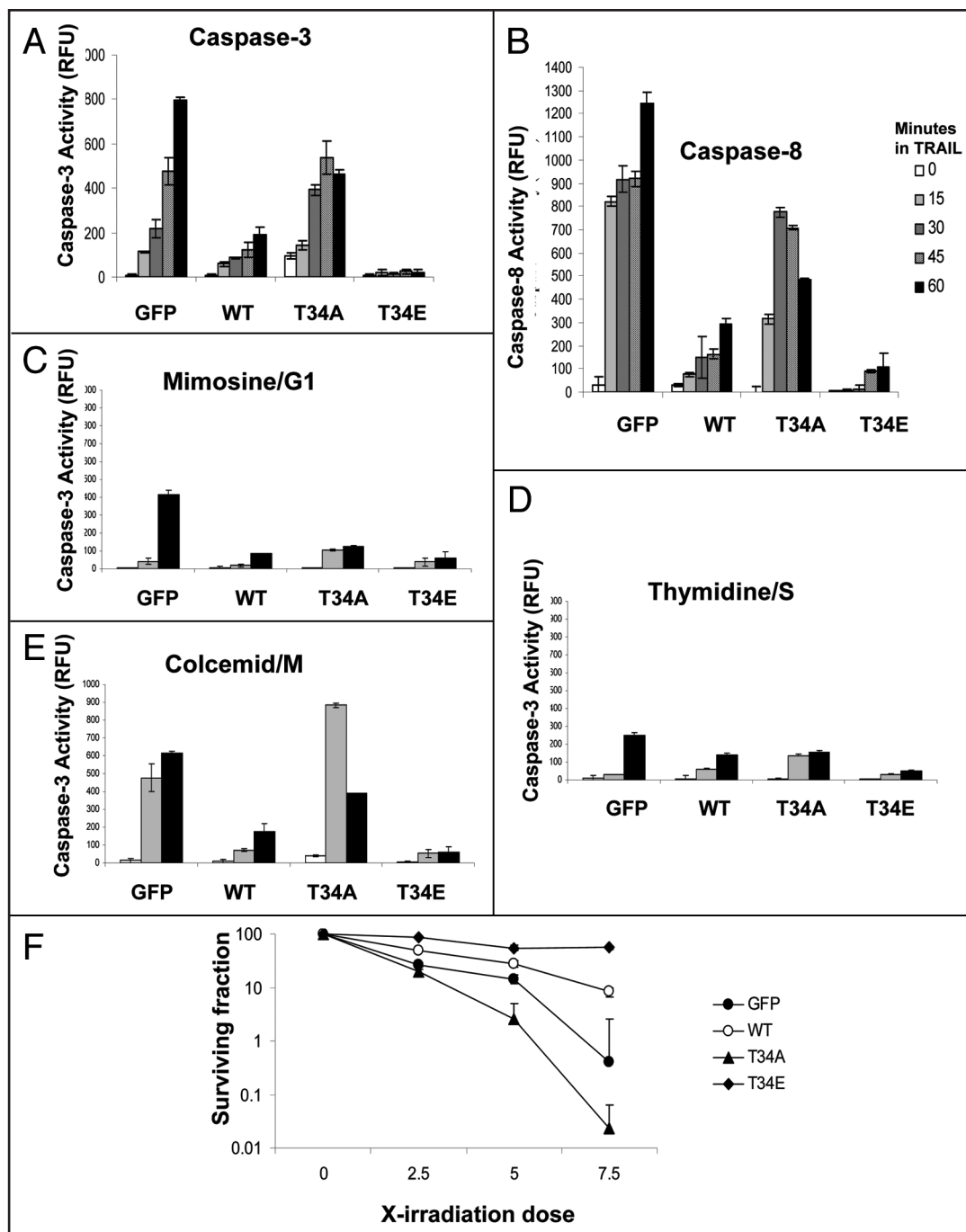


Figure 4. Mutation of T34 alters survivin's cytoprotection. (A) Apoptosis was induced in asynchronous cultures by exposure to TRAIL for the indicated times, lysates prepared and caspase-3 activity assessed by cleavage of the tetrapeptide Ac-DEVD-AMC. Apoptosis was induced more rapidly in survivin_{T34A}-GFP lysates than from control (GFP) cells. Survivin_{T34E}-GFP lysates almost completely inhibited apoptosis. (B) Caspase-8 activity was similar but occurred earlier. (C–E) Caspase-3 activity assays were performed in synchronised populations, arrested in G₁ (C), S-phase (D) or mitosis (E), after treatment with TRAIL for 0, 30 or 60 minutes (key as above). The most profound difference in survivin_{T34A}-GFP and survivin_{T34E}-GFP anti-apoptotic activities was seen in mitotic cells. (F) Cells were exposed to increasing doses of X-irradiation, allowed to grow for 7–9 days, and colonies of >50 cells counted. Surviving fractions are plotted logarithmically. Survivin_{T34A}-GFP cells conferred no resistance to X-rays, but survivin_{T34E}-GFP cells showed extraordinary resistance to this genotoxic stress.

saw only a modest elevation in the basal level of caspase-3 activity without TRAIL (Fig. 4A, time 0). Nevertheless, survivin_{T34A}-GFP greatly sensitizes cells to TRAIL, as apoptosis occurred more rapidly than in the control population. By far the most striking observation was the ability of survivin_{T34E}-GFP lysates to suppress, almost completely, TRAIL-mediated apoptosis.

The caspase-8 activity profiles were similar to those of caspase-3: caspase-8 activity peaked earlier, as expected for an “initiator” caspase (Fig. 4B).

As T34 is phosphorylated by cdk1 during mitosis,¹⁰ we considered whether inhibition of apoptosis was more apparent in mitosis than in other cell cycle stages. Cells were arrested in G₁ by 16 h

treatment with 500 μ M mimosine; S-phase by 16 h treatment with 2 mM thymidine; and M-phase by 16 h treatment with 0.2 μ g/ml colcemid, then exposed to TRAIL for 30 or 60 minutes, and caspase-3 activity assayed on the lysates (Fig. 4C–E). Caspase-3 activity was highest in mitotically arrested control and survivin_{T34A}-GFP cells, while survivin_{T34E}-GFP again ablated caspase-3 activity (Fig. 4E).

Next we exposed cells to increasing doses of X-irradiation and assessed clonogenic survival 7–9 days later. Cells expressing survivin-GFP were more resistant to irradiation than control (GFP) cells (Fig. 4F). This resistance was abolished by the mutation T34A. Strikingly, however, the phosphomimetic mutation, T34E, greatly enhanced resistance, with an astounding 55% of the population surviving a genotoxic insult of 7.5 Gy. Cytoprotection by survivin_{T34E}-GFP was previously observed after UV irradiation of melanoma cells,¹ however, as no comparison was made to wild type, the remarkable enhancement of survivin's anti-apoptotic activity by this pseudophosphorylation was not apparent. Augmentation of survivin's anti-apoptotic activity by cdk1-mediated phosphorylation parallels the regulation of caspase-9, which is inhibited from initiating apoptosis from mitosis when phosphorylated by cdk1.¹⁹ Thus it will be interesting to determine whether these proteins operate synergistically in promoting survival.

In conclusion, when survivin is unable to be phosphorylated at T34, growth is accelerated, but cells are not protected against death-inducing stimuli. In contrast, pseudophosphorylation of T34 retards growth, and does not support mitosis when expressed alone, however, it significantly enhances cell viability in response to death-inducing stimuli. Therefore survivin_{T34A}-GFP is dominant in proliferation, and dominant negative during apoptosis, while survivin_{T34E}-GFP is dominant negative in mitosis and dominant in apoptosis. Since survivin is phosphorylated on this residue *in vivo*, our data suggest that phosphorylation of T34 is a protective modification that preserves cell viability with incredibly high efficiency. Our data also suggest that like aurora-B,²⁰ cdk1 negatively regulates survivin during mitosis and T34-phosphorylation must be reversed, or the protein eliminated, in order for cells to complete division. Thus survivin's dual functions can be separated, and exaggerated, by modification of T34, which is pivotal in cellular decisions over life and death.

Materials and Methods

Unless otherwise stated, all chemicals were obtained from Sigma-Aldrich and tissue culture products from Invitrogen.

Cell lines. HeLa cell lines were established by FuGene-6 (Roche) mediated transfection of pcDNA3.1 constructs encoding GFP, survivin-GFP, survivin_{T34A}-GFP or survivin_{T34E}-GFP, followed by G418 selection and FACS sorting. Cells were cultured as described in Colnaghi et al. (2006). Cell proliferation assessed by trypan blue exclusion using a haemocytometer.

Fluorescence microscopy. Cells were fixed with 4% formaldehyde, permeabilized with 0.15% Triton then probed with primary antibodies: anti-borealin (1:200, in-house), anti-BubR1 (1:500, provided by Stephen Taylor), anti-phosphoH3^{ser10} (1:200, Upstate), or anti-tubulin (1:2000, B512), followed by Texas Red conjugated secondary antibodies (1:200, Vector Labs). All cells were counterstained with DAPI in Vectashield mounting medium (Vector Labs). Images were prepared as described in Colnaghi et al. (2006). To assess

the mitotic index, fixed cells were probed with anti-phosphoH3^{ser10} or anti-tubulin.

FACS profiling. Cells were harvested in ice cold PBS, fixed in 70% ethanol at -20°C for 2 h. They were then washed and treated with 100 μ g/ml RNase and stained with 100 μ g/ml propidium iodide in PBS to assess DNA content, and held at 25°C for 15 minutes prior to analysis. Samples were analysed using a FACS Canto (Becton Dickinson).

RNAi. Cells were seeded at 2×10^4 per well (24 well plate) in anti-biotic free media then transfected with 60 pmol of control or survivin specific siRNA (Ambion) using HyperFect (Qiagen), as described *in*.² Constructs were rendered resistant to RNAi-targeting by introduction of a silent mutation, G54C.^{20,21}

Immunoblotting. Immunoblot analysis was performed as described in Colnaghi et al. (2006). Antibodies used: anti-survivin (1:750, rabbit, Ab469, Abcam) and anti-tubulin (B512, 1:2000, Sigma). Signal intensity was detected using horseradish peroxidase conjugated secondary antibodies (1:2000; Dakocytomation), ECL and X-ray film (both G.E. Healthcare) or by Phosphoimaging (Storm).

Time lapse imaging. Cells were grown in 35 mm glass-bottomed Willco dishes in phenol-red free CO₂ independent medium (Invitrogen), supplemented as for DMEM. From 24–72 h post-transfection with siRNA cells were monitored at 20x using a Leica DMIRB inverted microscope with Simple PCI software (Digital Pixel). Images were prepared as JPEGs using Photoshop.

GST-pull down assays. Expression of GST-tagged proteins was induced in BL21 cells by the addition of 0.1 mM IPTG, and purified protein bound to glutathione sepharose 4B beads (G.E. Healthcare). *In vitro* translation was performed (90 minutes, 30°C) with pcDNA3.1 survivin, aurora-B or borealin using a TNT T7 kit (Promega), with ³⁵S methionine (G.E. Healthcare) as tracer. Beads and translated product were incubated for 1 h at 4°C, then analysed by SDS-PAGE and autoradiography.

TRAIL treatment and apoptosis assay. Cells were seeded at 5×10^4 per well in 24-well plates. The following day they were treated at 15 or 30 minute intervals with 250 ng/ml recombinant TRAIL (PeproTech EC Ltd.). Lysates were prepared in 200 μ l MPER buffer (Pierce) containing 1 mM EDTA, 1 μ g/ μ l pepstatin A, 1 mM AEBSF and centrifuged to remove debris. Caspase 3 activity was assessed spectroscopically by cleavage of the fluorogenic tetrapeptide substrate, Ac-DEVD-AMC (BioMol), as described previously.^{2,3} Caspase-8 activity was assessed similarly using Ac-IETD-AMC (BioMol).

Clonogenic survival assays. Cells were seeded at low density in Petri dishes and irradiated 2 h later at 0, 2.5, 5 or 7.5 Grays using an Hs-X-Ray System (A.G.O Installations Ltd., Reading, UK). Seven–ten days post-irradiation cultures were stained with methylene blue (1 h, 25°C), and colonies of >50 cells counted.

Statistical analysis. Comparisons among all groups were performed in Microsoft Excel with the one-way analysis of variance test. Values of $p < 0.05$ were considered significant.

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