VALIDATING SURVIVIN AS A CANCER THERAPEUTIC TARGET

Dario C. Altieri

Acquisition of the ability to evade cellular suicide, or apoptosis, is one of the master switches that contributes to cellular transformation and, ultimately, to invasive cancer. Much has been learned about the molecular organization of apoptotic pathways and their regulators, but the identification and validation of translational targets for apoptosis-based cancer therapy has posed a great challenge. Survivin is an attractive candidate for cancer therapy, so what is its potential applicability in the clinic?

RING FINGER
A protein domain that consists of two loops held together at their base by cysteine and histidine residues that complex two zinc ions. Many ring fingers function in protein degradation by facilitating protein ubiquitylation.

Department of Cancer Biology, LRB-428, University of Massachusetts Medical School, 364 Plantation Street, Worcester, Massachusetts 01605, USA.
e-mail: dario.altieri@umassmed.edu
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It has been almost three decades since apoptosis was identified as a cell-death phenotype with unique morphological characteristics[1]. It is now recognized as a genetic programme of cellular suicide that involves cascades of gene families, signalling pathways and specialized subcellular microenvironments. Apoptosis is essential for sculpting the developing organism during embryonic and fetal growth, removing unnecessary or outdated structures, and imparting plasticity to specialized tissue districts, such as the central nervous system[2]. In the adult organism, apoptosis maintains the homeostasis of differentiated tissues by regulating the balance between cell proliferation and cell death.

Apoptosis is mediated by caspases — a family of intracellular cysteine proteases that become activated by limited proteolysis and cleave cellular substrates that are involved in DNA repair, cytoskeletal organization, nuclear integrity and cell survival[3]. Mammalian cells use two main pathways to undergo apoptosis. An 'extrinsic' pathway is crucial for immune selection and inflammation, and is initiated by ligation of cell-surface trimeric death receptors, including the tumour necrosis factor-α (TNF-α) receptor and CD95 (FAS)[4]. An 'intrinsic' pathway is triggered by intracellular and environmental cues and is centred on dysregulation of mitochondrial function[5]. This culminates in an increase in mitochondrial membrane permeability, and release in the cytoplasm of proteins that facilitate caspase activation, most notably cytochrome *c*[6] and SMAC/DIABLO[7] (see below) (FIG. 1). Both pathways transduce the apoptotic signal by the formation of multimeric complexes[4,5], which favour proteolytic activation of initiator caspases (FIG. 1).

Two gene families of apoptosis regulators have been identified. The BCL2 family comprises molecules with pro- or anti-apoptotic function, which are identified by 1–4 copies of a BH (BCL2 homology) domain and a carboxy-terminal hydrophobic region[8]. BCL2 proteins decrease (anti-apoptotic; that is, BCL2 and BCL-X_L) or enhance (pro-apoptotic; that is, BAX, BAD, BAK and BID) mitochondrial permeability, and particularly determine whether cytochrome *c* is released[8] (FIG. 1). Several models for the function of BCL2 have been proposed, including physical association with mitochondrial channels or direct ability to act as pore-forming structures. The central feature of BCL2 molecules is their ability to homodimerize or heterodimerize at the mitochondrial membrane, and it is the differential recruitment of pro- or anti-apoptotic family members that is thought to tilt the balance between cell death and cell survival[8]. Among pro-apoptotic BCL2 family members, the 'BH3-only' molecules BAX and BAK might be required to initiate most, if not all, mitochondrial-dependent apoptosis[9] (FIG. 1).

Another family that regulates cell death is the inhibitor of apoptosis (IAP) proteins[10]. IAPs contain 1–3 copies of a 70-amino-acid zinc-finger fold, which is designated baculovirus IAP repeat (BIR). Other structural features that are found in certain IAPs include a caspase-recruitment domain (CARD), and a carboxy-terminal RING FINGER[10]. In humans, eight members of the IAP gene family have been identified

Summary

- In mammalian cells, apoptosis is modulated by two protein families — the BCL2 and inhibitor of apoptosis (IAP) families.
- Survivin is a unique member of the IAP family. It is associated with several subcellular compartments and its expression is regulated by many signalling pathways.
- The survivin pathway interfaces with both the cell-death machinery and mechanisms of cell-cycle progression and microtubule stability.
- Survivin expression is undetectable in most normal adult tissues, but is overexpressed in virtually every human tumour that has been studied. Several mechanisms have been proposed to account for this overexpression, one of which is loss of wild-type p53.
- Is survivin a rational target for cancer therapy? Using molecular antagonists of survivin is one approach for enhancing cell death — specifically of tumours — and could be used in combination with conventional chemotherapy- or radiation-based treatments.

— including the most studied XIAP, c-IAP1, c-IAP2 and survivin — and similar proteins have been found in the genomes of yeast, flies and worms. IAPs counteract apoptosis by acting as endogenous inhibitors of caspases, so target a step that is downstream of BCL2 (REF. 10) (FIG. 1). This mechanism has been elucidated in detail with respect to the kinetics of enzyme inhibition, assembly of IAP–caspase complexes and the structure–function requirements of caspase recognition[11]. Proteins that are released by mitochondria during changes in permeability — including SMAC/DIABLO and OMI/HTRA2 — bind to IAPs and relieve their inhibition of caspases[11]. The apoptosis-inhibitory function of IAPs is evolutionarily conserved, and *Drosophila* homologues of IAPs are essential for cell survival[10]. Other mechanisms by which IAPs can exert cytoprotection might involve activation of TGF-β or c-JUN amino-terminal kinase (JNK) signalling[12,13].

Certain IAPs have also been implicated in cell division. This first emerged in gene-targeting studies in yeast[14–16] and *Caenorhabditis elegans*[17,18], in which IAP ablation was not associated with cell-death aberrations but resulted in meiotic and mitotic defects with inappropriate chromosomal segregation, failed cytokinesis and polyploidy. The mechanism by which IAPs participate in cell division in these lower organisms is still unclear, but pleiotropic functions are likely, and might include control of spindle elongation, sister-chromatid separation and cytokinesis.

In mammalian cells, deletion of certain IAP genes has been unremarkable, potentially because of redundancy by other family members[19]. On the other hand, there is evidence that IAPs have an important role in preserving a steady-state anti-apoptotic environment in cells[10]. Accordingly, rapid changes in IAP expression — as controlled by ubiquitylation and proteasome-dependent destruction[20] — resulted in catastrophic effects on cell viability, with spontaneous induction of apoptosis and an enhanced response to cell-death-inducing stimuli[21]. Destroying IAPs as a means of enhancing apoptosis is an evolutionarily conserved mechanism, and a *Drosophila* death-inducing gene, *Hid*, mediates ubiquitylation and proteasome destruction of an IAP homologue, DIAP1

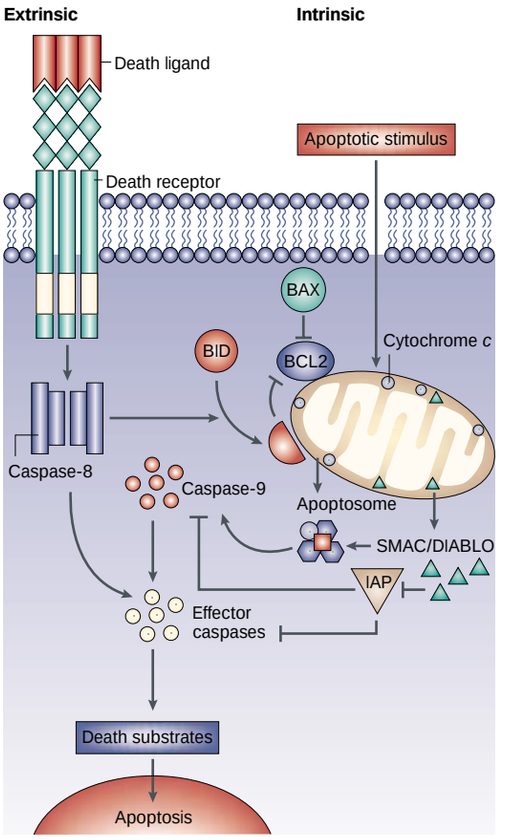

Figure 1 | **Apoptotic pathways and their regulators.** Apoptosis can be initiated by the death-receptor (extrinsic) pathway that acts through caspase-8 or mitochondrial (intrinsic) pathway that acts through caspase-9, but both pathways converge to activate the effector caspases, which act on the death substrates. In addition, cell death is regulated by the BCL2 and inhibitor of apoptosis (IAP) protein families. BCL2 proteins are thought to regulate the mitochondria permeability transition by inhibiting (BCL2 and BCL-X_L) or promoting (BAX and BID) cytochrome *c* release, whereas IAP proteins act downstream to prevent processing of initiator caspase-9 from the apoptosome — a supramolecular caspase-activating complex that also contains cytochrome *c* and apoptosis activating factor 1 (APAF1) — and inhibiting the activity of the effector caspases. Proteins that are released by mitochondria during the permeability transition, including cytochrome *c* and SMAC/DIABLO, facilitate caspase activation by forming the apoptosome or relieving the caspase-inhibitory function of IAP proteins, respectively.

TGF-β
A ligand that activates members of a superfamily of cell-surface receptors that include the bone morphogenic protein (BMP) receptors; after ligand activation, the signal is transduced, mainly through the SMAD family of transcription factors and co-activators.

JNK
(c-JUN amino-terminal kinase). A stress-induced protein kinase that has been implicated in cell death or cell viability, depending on the cellular context, and is activated by XIAP.

(REF. 22). Conversely, there is also evidence that the E3 ligase activity that is embedded in the RING domain of certain IAPs — such as XIAP and c-IAP2 — might contribute to cytoprotection and facilitate proteasome-dependent destruction of bound caspase-3 and -7 (REFS 20,23).

Structure and subcellular distribution of survivin

At 16.5 kDa, survivin is the smallest member of the mammalian IAP family; it carries a single BIR and an extended carboxy-terminal α-helical coiled-coil, but no RING finger or other identifiable domain[24]. A single

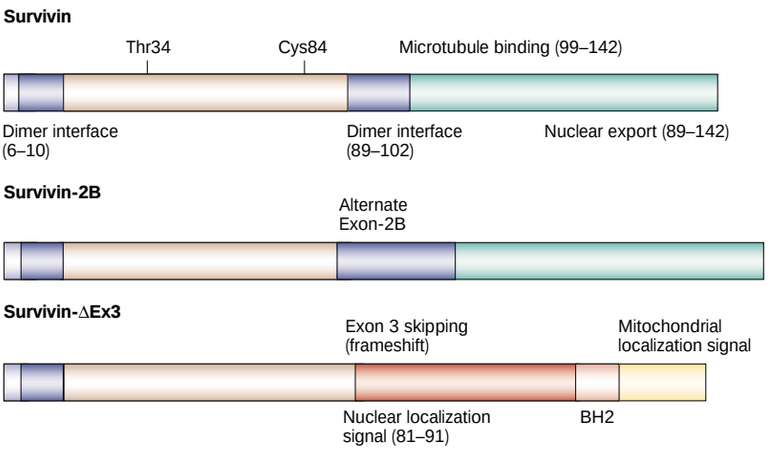

Figure 2 | **Structure–function of survivin proteins generated by alternative splicing.** Organization of survivin and its alternatively spliced variants, involving insertion of an alternative exon (survivin-2B) or deletion of exon 3 (survivin-ΔEx3). Discrete regions implicated in dimerization, microtubule binding, nuclear localization/export and subcellular targeting to mitochondria are indicated.

copy of the *survivin* gene gives rise to three alternatively spliced transcripts (FIG. 2). In addition to wild-type survivin, which exhibits a three-intron–four-exon structure in the human[25] and mouse[26] genomes, two survivin isoforms are generated by insertion of an alternative exon 2 (survivin-2B) or removal of exon 3 (survivin-ΔEx-3). The latter results in a frameshift and generates a unique carboxyl terminus sequence of potential functional relevance[27] (FIG. 2). In overexpression studies, survivin-ΔEx-3 showed a selective nuclear accumulation that could involve, at least in part, a bipartite nuclear localization signal that is embedded in the new carboxyl terminus[28]. Other unique features in survivin-ΔEx3 were also identified, including a mitochondrial localization signal and a BH2 domain (FIG. 2). These were reminiscent of a survivin-ΔEx3 homologue that was identified in the herpes simplex virus (HSV) genome; this localized to mitochondria and inhibited apoptosis by associating with BCL2 and by suppressing caspase-3 activity by a mechanism that was dependent on the BIR[29]. X-ray crystallography of human[30,31] or mouse[32] survivin has shown that they are organized as stable homodimers — a configuration that is probably common to other IAP proteins such as XIAP.

Whereas the timing of expression of other IAPs has not been extensively investigated, survivin shows a clear cell-cycle-dependent expression at mitosis. This is largely controlled at the transcriptional level, and involves canonical CDE/CHR (cell-cycle-dependent element/cell-cycle gene homology region) boxes[33] that are located in the proximal survivin promoter[34,35] (FIG. 3a). However, changes in protein stability also contribute to survivin accumulation at mitosis. Accordingly, polyubiquitylation of survivin and proteasome-dependent destruction have been demonstrated in interphase cells[36], and mitotic phosphorylation of survivin on Thr34 by CDC2–cyclin-B1 has been associated with increased protein stability at metaphase[37] (FIG. 3a). When expressed at mitosis, survivin has been shown to localize to various components of the mitotic apparatus, such as centrosomes, microtubules of the metaphase and anaphase spindle, and the remnants of the mitotic apparatus — midbodies[25,38–41]. A direct association between survivin and polymerized tubulin has been demonstrated *in vitro*[25]; this mapped to the survivin carboxyl terminus, which might contribute to its localization to the mitotic apparatus (FIG. 2). A subcellular pool of survivin has also been localized to kinetochores of metaphase chromosomes[40], potentially associating with the multifunctional mitotic regulator Aurora B kinase[42]. The subcellular pools of survivin are immunochemically distinct, potentially reflecting post-translational modifications and/or differential epitope accessibility[41]. Quantitatively, however, and at variance with preliminary immunostaining studies[43], survivin is largely excluded from the nucleus[41]. This might involve a specific pathway of CRM1-mediated nuclear export — identified in mutagenesis experiments and for which the survivin carboxyl terminus is required[28].

Other non-cell-cycle-dependent mechanisms contribute to survivin expression (FIG. 3b). These have been predominantly demonstrated in non-transformed cells, as opposed to cancer cell lines. First, stimulation of quiescent $CD34^+$ bone-marrow-derived stem cells with haematopoietic cytokines resulted in increased survivin expression in the absence of cell-cycle progression, as determined by lack of Ki67 or cyclin D expression and by hypophosphorylated retinoblastoma (RB)[44,45]. Second, the inflammatory or vascular remodelling cytokines interleukin (IL)-11 (REF. 46) and angiopoietin-1 (REF. 47) increased survivin expression in endothelial cells independently of cell-cycle progression, in a pathway that required phosphorylation of signal transducer and activator of transcription 3 (STAT3) or phosphatidylinositol 3-kinase (PI3K)/AKT SIGNALLING. A STAT3 requirement for *survivin* gene expression has been independently shown in lymphoma, in which apoptosis that is induced by STAT3 inhibition was counteracted by *survivin* expression[48].

Apoptosis inhibitor, mitotic regulator, or both?

Because of its unique properties, the question as to whether survivin functioned in cytoprotection, as with other mammalian IAPs, or in the regulation of cell division, as with BIR-containing IAPs in yeast and *C. elegans*, has frequently been asked. This has proved controversial[49,50], although a role of survivin in apoptosis inhibition is now well established.

In cell culture systems, overexpression of *survivin* has been consistently associated with inhibition of cell death that was initiated by both the extrinsic and intrinsic apoptotic pathways[24,27,34,51–57]. Transgenic animals that expressed survivin in the skin also suppressed apoptosis that was induced by ultraviolet B (UVB) irradiation[58], and livers that were isolated from heterozygous *survivin* animals showed low levels of spontaneous apoptosis that was characterized by BAX accumulation, cytochrome *c* release and exaggerated cell death that was induced by suboptimal FAS

Ki67
A monoclonal antibody that marks the late S phase of the cell cycle. This is frequently used to mark proliferating cells in tissues or cell suspensions.

PI3K/AKT PATHWAY
The phosphatidylinositol 3-kinase (PI3K) family of enzymes are activated in response to a wide variety of stimuli and catalyse the phophorylation of inositol lipids at the D-3 position of the inositol ring. These phosphoinositides act as second messengers; a primary target is the serine/threonine kinase AKT (protein kinase B). Activated AKT phosphorylates several cellular targets, including proteins that are involved in cell survival, proliferation and migration.

STAT3
A member of the STAT (signal transducer and activator of transcription) family of transcription factors. STATs are activated through phosphorylation by Janus kinases and have an important role in cytokine-receptor signalling.

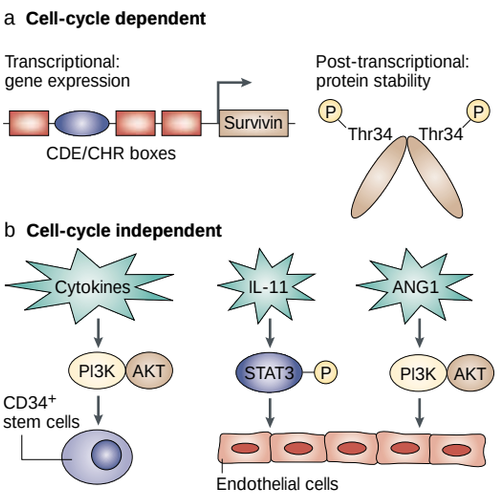

Figure 3 | **Differential regulation of survivin expression in cell-cycle-dependent and -independent pathways.** **a** | Transcriptional and post-transcriptional mechanisms that control survivin expression involve CDE/CHR (cell-cycle-dependent element/cell-cycle gene homology region) G1 repressor elements in the *survivin* promoter and increased protein stability by phosphorylation on Thr34, respectively. **b** | Cell-cycle-independent mechanisms that influence survivin expression levels have been shown to involve response to haematopoietic and vascular remodelling cytokines, STAT3-dependent signal transduction and phosphatidylinositol 3-kinase (PI3K) activity. ANG1, angiopoietin 1; IL-11, interleukin-11; STAT3, signal transducer and activator of transcription 3.

ligation[59]. When expressed in the thymus, transgenic survivin did not affect thymocyte apoptosis but favoured increased cellularity in response to selected stimuli, indicating that it could create a permissive survival environment at cell division[60]. Finally, targeting survivin with ANTISENSE, RIBOZYMES or expression of DOMINANT-NEGATIVE MUTANTS resulted in caspase-dependent cell death, an enhancement of cell-death stimuli and suppression of tumour growth *in vivo*[25,61–68]. The apoptosis-inhibitory function of survivin is evolutionarily conserved, and *Deterin* — a *Drosophila* survivin homologue — functions interchangeably with survivin to block apoptosis[69] in mammalian or insect cells[39].

Although the notion that survivin inhibits apoptosis is established, the mechanism(s) by which this occurs has not been conclusively determined. As with other IAPs, a physical interaction between survivin and initiator or effector caspases has been reported by several groups[34,51,54]. However, with the exception of two published studies[70,71], this did not seem to translate into physiologically meaningful inhibition of caspase activity[30,54]. In addition, the crystal structure of survivin does not reveal the presence of a 'hook and sinker' region that mediates caspase binding in other IAPs[30]. Recent data point to a more selective role of survivin in antagonizing mitochondrial-dependent apoptosis. This reflects the ability of survivin to block mitochondrial-induced, but not death-receptor-induced, apoptosis in transgenic animals[58]; to associate with caspase-9 (REF. 72) and SMAC/DIABLO[7]; and to localize to mitochondria at least under certain apoptosis-inducing conditions[59]. Similarly, cell death following loss/interference of survivin has the characteristics of mitochondrial-dependent apoptosis, such as cytochrome *c* release[73], caspase-9 activation[72] and requirement of apoptosome components[5,37]. Recent genetic evidence is consistent with this view, and heterozygous *survivin* mice showed increased sensitivity to mitochondrial-dependent cell death[59] (BOX 1).

In addition to cell-death regulation, there is also strong experimental evidence that survivin is important in cell division. This first surfaced in targeting experiments with antisense technology or dominant-negative mutants, which resulted in a dual phenotype of apoptosis and aberrant mitotic progression, with supernumerary centrosomes, multipolar mitotic spindles, failed cytokinesis and multinucleation[38,63,74]. The phenotype of *survivin*-knockout mice is consistent with a crucial role of survivin in mitosis; survivin is indispensable during embryonic development[43]. Beginning at embryonic day (E) 2.5, homozygous deletion of the *survivin* gene resulted in a catastrophic defect of microtubule assembly, with absence of mitotic spindles, formation of disorganized tubulin aggregates and multinucleation, leading to 100% lethality by E 3.5–4.5 (REF. 43). An identical phenotype has been observed independently, and confirmed on three different mouse genetic backgrounds[59]. How survivin participates in mitosis has not been conclusively determined. At least a minor pool of human survivin has been found to associate with INCENP and Aurora B kinase[42] — molecules that are involved in late-stage mitosis and cytokinesis — and the interaction between a *Xenopus laevis* survivin-like molecule and Aurora B produced a tenfold increase in Aurora B's kinase activity in a cell-cycle-dependent manner[75]. A similar model has been recently proposed for human survivin. Experiments in which survivin was targeted by microinjection or lipid-based intracellular antibody delivery, however, indicated a broader role of survivin in several phases of mitosis. In these studies, antibody targeting of survivin resulted in premature sister-chromatid separation, dysregulation of spindle-checkpoint activation[74], sustained metaphase arrest frequently followed by apoptosis[76] and formation of multipolar spindles[41]. By immunofluorescence, cells that had been microinjected with an antibody to survivin had short mitotic spindles that were severely depleted of microtubules. Conversely, retroviral or adenoviral expression of survivin preserved spindle stability and dynamics against microtubule poisons, and this promoted cell viability and resistance to chemotherapy in tumour cells[76] and endothelial cells[77] (BOX 1).

Differential expression of survivin in cancer

One of the most significant features of survivin is its differential expression in cancer versus normal tissues[24]. Survivin is strongly and diffusely expressed in embryonic and fetal organs[34,78], but undetectable in

ANTISENSE TECHNOLOGY
This uses an oligonucleotide that is complementary to a portion of mRNA. It binds to the mRNA and arrests translation by physical blockade of ribosomal machinery and/or by activation of endogenous RNases.

RIBOZYMES
RNA molecules that function like enzymes and exert a catalytic activity. Ribozymes can be designed to cleave specific mRNAs and thereby inhibit protein synthesis.

DOMINANT-NEGATIVE MUTANTS
A non-functional mutant protein that competes with the normal, non-mutated protein, thereby blocking its activity.

TRANSCRIPTOME
mRNA transcripts identified by serial analysis of gene expression (SAGE) that are selectively expressed in human tumours but undetectable or found at very low levels in normal tissues that are isolated from the same organs.

WNT–β-CATENIN SIGNALLING PATHWAY
A developmental pathway of key importance for patterning and specification of body axes in embryogenesis via activation of genes that are mediated by the T-cell factor (TCF) group of transcription factors. Deregulated signalling through WNT/TCF/β-catenin has been implicated in a variety of human tumours, most notably colon cancer, potentially by deregulating the balance between cell proliferation/cell differentiation in stem-cell compartments.

APC
Germ-line mutations of the adenomatous polyposis coli (*APC*) tumour-suppressor gene cause familial adenomatous polyposis — a genetic disorder that is characterized by an increased predisposition to colorectal cancer.

Box 1 | Survivin functions

Mitotic regulator
- Localization to the mitotic apparatus
- Catastrophic defect of mitosis in *survivin*$^{-/-}$ embryos
- Failed cytokinesis and polyploidy induced by antisense or dominant-negative mutants
- Defects in spindle assembly, chromatid separation and spindle-checkpoint activation after antibody microinjection

Cell-death inhibitor
- Inhibition of extrinsic/intrinsic apoptotic pathways
- Resisitance to apoptosis in transgenic mice
- Increased sensitivity to apoptosis of *survivin*$^{+/-}$ mice
- Spontaneous apoptosis induced by antisense, dominant-negative mutants or ribozyme
- Association with caspases and SMAC/DIABLO

most terminally differentiated normal tissues[24]. Adult normal cell types that express survivin include thymocytes[24], CD34^{+} bone-marrow-derived stem cells[44,79] and basal colonic epithelial cells[80,81]. In the mouse, strong expression of survivin in the gonads has also been reported. Although a common denominator among survivin-positive normal cells is their proliferative potential, survivin has not been detected in keratinocytes of the basal layer of the skin[58]. By contrast, dramatic overexpression of survivin was shown in tumours of lung[82], breast[83], colon[84], stomach[85], oesophagus[86], pancreas[87], liver[88], uterus[89], ovaries[90], Hodgkin's disease[91], non-Hodgkin's lymphoma[92,93], leukaemias[94,95], neuroblastoma[53,96], phaeochromocytoma[97], soft-tissue sarcomas[98], gliomas[99] and melanoma[62]. The normal tissues from these same organs did not express survivin. In gene-profiling studies, survivin was identified as the fourth 'TRANSCRIPTOME' expressed in the most common human cancers, but not in normal tissues. The two alternatively spliced forms of survivin show a comparable differential expression in cancer, but not in normal tissues[100].

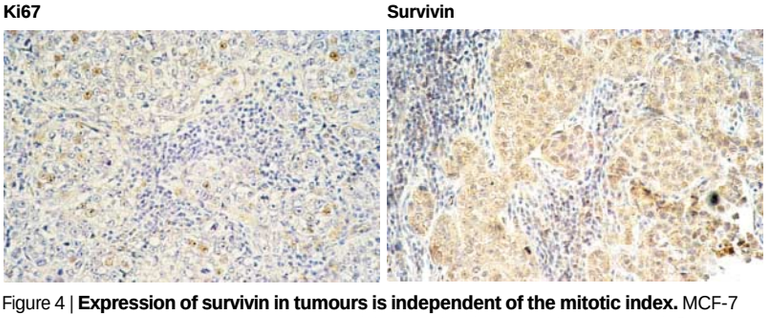

Figure 4 | **Expression of survivin in tumours is independent of the mitotic index.** MCF-7 human breast cancer xenografts were excised, formalin fixed and paraffin embedded. Samples were analysed with antibodies to Ki67 or survivin by immunohistochemistry. Survivin expression is typically seen in most tumour cells, and vastly exceeds the percentage of cycling cells that are identified by Ki67 labelling, which indicates that expression of the *survivin* gene is deregulated in cancer, and is actively transcribed in all cell-cycle phases.

The molecular basis for the overexpression of survivin in cancer has been intensely investigated. As *survivin* gene transcription is linked to mitotic progression, it was originally thought that the overexpression that is seen in cancer simply reflected a higher number of proliferating cells that were present in malignant tissues, and that the proportion of cycling, potentially survivin-positive cells in normal tissues was below the limit of experimental detection. However, compelling experimental evidence argues against this hypothesis. First, comparable survivin expression was found in tumours regardless of their mitotic indices[62], and, second, survivin reactivity is typically observed in the vast majority of tumour cells *in vivo*, in excess of the number of cycling cells that are detected, for instance, by Ki67 reactivity (FIG. 4). This has prompted the alternative possibility that the *survivin* gene might be globally deregulated in transformed cells, so that survivin is overexpressed in all cell-cycle phases, not just mitosis. Consistent with this view, negligible survivin promoter activity was detected in primary cell types as opposed to tumour cell lines[26], and cells transfected with a reporter gene placed under the control of a *survivin* promoter showed selective cancer-specific activity *in vitro* and *in vivo*[101]. Several molecular lesions associated with cancer have been implicated in the potential deregulation of *survivin* gene expression, including amplification of the *survivin* locus on 17q25 in neuroblastoma[53], selective demethylation of survivin exon 1 in ovarian cancer but not in normal ovaries[102], and, importantly, loss of wild-type p53. Three groups have independently identified *survivin* as one of the genes that is transcriptionally repressed by wild-type p53, potentially through a bipartite p53-responsive element in the *survivin* promoter, or changes in chromatin structure that affect promoter accessibility[55,56,68]. Functionally, *survivin* expression counteracted p53-dependent apoptosis[56], and loss of *survivin* by wild-type p53 contributed, at least in part, to p53-dependent cell death[55,68]. Finally, *survivin* was identified as a potential target gene of the WNT–β-CATENIN SIGNALLING PATHWAY[81], and upregulation of *survivin* in colorectal cancer has been speculated to result from *APC* mutations and aberrant stabilization of β-catenin[81].

In retrospective trials, patients whose tumours expressed survivin had a decreased overall survival[82–84,86,92,98,99,103–105], an increased rate of recurrence[106], a resistance to therapy[57,77] and a reduced apoptotic index *in vivo*[83,84,87]. Intuitively, this is in keeping with the dual role of survivin in apoptosis control and preservation of mitotic progression, indicating that overexpression of survivin might confer growth and survival advantages for tumour onset and progression. Consistent with this model, survivin expression seems to occur early during stepwise tumorigenesis, and elevated levels of survivin are present in skin premalignant lesions, indistinguishably from survivin expression in full-blown tumours[62].

Validating the survivin pathway for cancer therapy

The two main reasons for considering survivin as an attractive therapeutic target in cancer are its differential expression in tumours versus normal tissues, and its potential requirement for maintaining cancer-cell

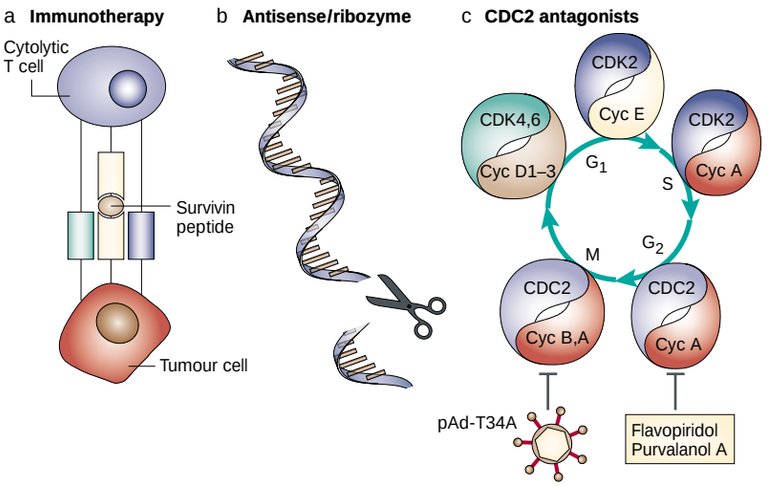

Figure 5 | **Current approaches for survivin targeting in cancer therapy. a** | Generation of antigen-specific cytolytic T cells against survivin peptides is being considered for vaccination strategies. **b** | Molecular antagonists of survivin, including antisense, ribozymes and expression of dominant-negative mutants, have been consistently associated with induction of apoptosis, enhancement of cell-death stimuli and inhibition of tumour growth *in vivo*. **c** | Interference of mitotic phosphorylation of survivin by CDC2 by sequential administration of cyclin-dependent kinase inhibitors flavopiridol or Purvalanol A after chemotherapy-induced mitotic arrest has resulted in strong anticancer activity *in vitro* and *in vivo*. A similar response has been observed by adenoviral overexpression of a phosphorylation-deficient survivin Thr34→Ala mutant (pAd-T34A).

viability. A survivin-based therapy would be expected to carry limited toxicity for normal tissues and to be effective at removing a general cell-viability machinery that is exploited by cancer cells. A promising therapeutic approach to target survivin relied on the ability to generate an antigen-specific immune response against survivin-bearing tumour cells[107] (FIG. 5a). This hypothesis has been independently validated by several groups by the observation that T cells mount a vigorous CYTOLYTIC RESPONSE against survivin peptides *in vitro* and *in vivo*[100,108–111], and that HLA CLASS I-RESTRICTED CYTOLYTIC T CELLS against survivin peptides exist in patients with breast cancer, leukaemia and melanoma *in vivo*[111]. These data indicate that a cancer-specific immune response to survivin might be used for potential vaccination strategies, with the advantage of minimizing the risks of autoimmune effects[107] (FIG. 5a).

A second strategy to interfere with the survivin pathway involved the use of 'molecular antagonists'. Transfection of antisense or ribozymes to ablate expression of endogenous *survivin* mRNA has consistently shown promising results in several independent studies (FIG. 5b). In these experiments, loss of *survivin* expression was sufficient to trigger caspase-dependent apoptosis in tumour cell lines, but not in normal cells, to enhance chemotherapy-induced cell death, to dysregulate mitotic progression with inhibition of cell proliferation, and to promote tumour eradication alone or when combined with immunotherapy or anticancer treatment *in vivo*[38,39,54,61,63–66,68,112]. Similar results were obtained by overexpressing survivin mutants, and two have been characterized for what seems to be a dominant-negative effect (see FIG. 2 for amino acids).

The first mutation disrupts the Zn^{2+} coordination sphere by targeting the highly conserved Cys84 in the third β-strand of the survivin BIR[25]. Forced expression of the survivin Cys84→Ala (survivin-C84A) mutant resulted in increased caspase activity in cells traversing mitosis[25], spontaneous apoptosis of various tumour cell types[25,62], reversal of cytoprotection in angiogenically (vascular endothelial growth factor (VEGF))-stimulated endothelial cells[77] and enhancement of antitumour immunotherapy *in vivo*[66]. It is unclear how survivin-C84A functions as a dominant-negative mutant; it binds to polymerized tubulin *in vitro* and localizes to spindle microtubules — demonstrated by confocal microscopy — indistinguishably from wild-type survivin[25]. However, protein-turnover experiments showed that survivin-C84A was unstable and had accelerated degradation by means of ubiquitin-dependent proteasome destruction[36]. Therefore, a potential dimerization of survivin-C84A with endogenous survivin might promote accelerated degradation of the complex, so reducing survivin levels below a crucial anti-apoptotic threshold.

The second mutation that has been used for gene-targeting experiments is Thr34→Ala. Thr34 was identified in the survivin crystal structure as a predicted site of phosphorylation by the mitotic kinase CDC2–cyclin-B1 (REF. 72). Indeed, CDC2–cyclin-B1 was shown, *in vivo*, to physically associate with survivin and to phosphorylate survivin on Thr34 during mitosis. A direct link between survivin and CDC2 was independently confirmed using array technology in gene-profiling studies of large-cell non-Hodgkin's lymphoma; it was associated with an activated B-cell phenotype and unfavourable disease progression[93]. The rationale of targeting Thr34 phosphorylation for therapeutic intervention came from experiments using a phosphorylation-defective survivin Thr34→Ala mutant (survivin-T34A). Conditional expression or adenoviral delivery of survivin-T34A (FIG. 5c) initiated massive mitochondrial-dependent apoptosis in a variety of tumour cell lines, showed no toxicity for normal cells and suppressed tumour growth in breast cancer and melanoma xenograft models *in vivo*[72,73,113]. Conversely, expression of a phosphorylation-mimetic survivin Thr34→Glu mutant strongly inhibited p53-induced apoptosis[56]. In protein-turnover experiments, survivin-T34A showed a 4–5-fold accelerated clearance compared with wild-type survivin, indicating that phosphorylation on this site might also contribute to protein stability at mitosis[37]. As with the model of survivin-C84A, accelerated degradation of a survivin–survivin-T34A complex might result in a dominant-negative effect by lowering endogenous survivin levels.

If CDC2 phosphorylation of survivin on Thr34 is required for cancer-cell viability, then it should be possible to target this step with a more generally applicable pharmacological approach. Kinase inhibitors, including antagonists of cyclin-dependent kinases (CDKs), have recently emerged as promising anticancer drugs for their ability to disrupt a number

CYTOLYTIC RESPONSE
Destruction of cellular targets by T lymphocytes via a perforin–granzyme mechanism or FAS–FAS-ligand interaction, leading to the dissolution of membrane integrity.

HLA CLASS I-RESTRICTED CYTOLYTIC T CELLS
Antigen-specific T cells recognizing peptides presented through the HLA class I major histocompatibility complex are capable of lysing compatible target cells. These cells might belong to the CD4 or CD8 subset and are believed to act in immune surveillance against tumours.

LOSS OF HETEROZYGOSITY (LOH). In cells that carry a mutated allele of a tumour-suppressor gene, the gene becomes fully inactivated when the cell loses a large part of the chromosome carrying the wild-type allele. Regions with high frequency of LOH are believed to harbour tumour-suppressor genes.

of cell-proliferation/cell-survival pathways[114]. In particular, flavopiridol — a flavone inhibitor of several CDKs, including CDC2 — induces apoptosis in various tumour cell types, and is now being explored in the clinic as an anticancer agent[114,115]. Consistent with this view, there is also genetic evidence to indicate that endogenous inhibitors of CDC2 might function as tumour suppressors; disruption of the human homologue of the *Drosophila lats* gene has been associated with deregulated CDC2 kinase activity, increased cell proliferation and tumour formation[116]. LOSS OF HETEROZYGOSITY of the human *LATS1* locus has been shown in human tumours[117], whereas forced expression of human *LATS1* suppressed CDC2 kinase activity and induced apoptosis[118]. A cytoprotective role of CDC2 kinase was also indicated by conditional knockout experiments, in which deletion of both *CDC2* alleles resulted in apoptosis and DNA endoreduplication[119], in agreement with earlier cell-based experiments[120].

The possibility of using CDC2 antagonists to block survivin phosphorylation on Thr34 and recapitulate the anticancer phenotype that is induced by survivin-T34A[72,73,113] has recently been investigated (FIG. 5c). The rationale was to use the chemotherapeutic taxol — a microtubule poison that induces mitotic arrest with elevated CDC2 kinase activity and hyperphosphorylation of survivin on Thr34 (REF. 57) — followed by administration of the CDK inhibitor. This is relevant to the role of survivin as an antagonist of taxol-induced apoptosis[25] and a chemoresistance factor for taxane-based regimens in patients with ovarian cancer[57]. In these experiments, flavopiridol or a more selective second-generation CDC2 inhibitor, Purvalanol A (Purv. A)[121], inhibited survivin phosphorylation on Thr34 *in vitro* and *in vivo*, which dramatically enhanced apoptosis induced by low-dose taxol, and blocked tumour growth in a breast cancer xenograft model *in vivo*[37]. The strict sequence dependence of this regimen for anticancer activity (taxol first, and CDK antagonist second) is consistent with the reported clinical efficacy of taxol–flavopiridol sequential combination emerging in recent trials in solid tumours[115], and might provide some molecular clues as to the role of CDC2 in cancer-cell survival. When administered in the reversed order, the combination of Purv. A followed by taxol not only failed to promote apoptosis, but paradoxically enhanced tumour growth *in vivo*[37]. This is reminiscent of previous observations in which inhibition of CDC2 by upregulating WAF1 (also known as p21 and CIP1)[122] actually attenuated apoptosis induced by microtubule poisons. One potential explanation is that premature suppression of CDC2 kinase might arrest mitotic progression before cells require the enhanced anti-apoptotic environment mediated by survivin that is phosphorylated on Thr34.

Despite the promising data in xenograft models, it is still unclear how survivin targeting by antisense or dominant-negative mutants blocks tumour growth. The most intuitive hypothesis is that interference/loss of survivin might result in selective apoptosis of the proliferating tumour-cell compartment — a model that is supported by the kinetics of apoptosis in cell-cycle-synchronized cultures[72]. However, a possibility that is not mutually exclusive is that survivin targeting might also compromise endothelial-cell viability and interfere with tumour angiogenesis. This is consistent with the prominent induction of survivin in endothelial cells that is observed during the proliferative[123,124] or remodelling[47] phases of angiogenesis and is associated with resistance to apoptosis[123]. Conversely, antisense ablation of survivin during angiogenesis removed the cytoprotective effect of VEGF, caused endothelial-cell apoptosis and promoted rapid involution of three-dimensional capillary-like vessels *in vitro*[125]. The role of survivin in endothelial-cell protection might also have a crucial role in promoting chemoresistance during taxol treatment[77].

Concluding remarks and future directions

Compelling experimental evidence from several areas of investigation has identified a crucial role for survivin in the preservation of cell viability and the maintenance of normal mitotic progression. The original premise that this pathway was exploited in many human cancers, but was undetectable or present at low levels in normal tissues[24], was intensely scrutinized, and has now been unanimously validated. A second hypothesis that, despite the redundancy of cell-death and cell-proliferation pathways, survivin could be required for cancer-cell viability was also confirmed using cellular, molecular and genetic approaches. These credentials indicate that survivin could, in principle, be a rational target for cancer therapy. Apoptosis-based cancer therapy is still in its infancy, but its underlying premise has passed proof of principle, and early clinical trials have provided limited but encouraging initial results[126]. Emerging translational opportunities have now focused on survivin for potential therapeutic testing, ranging from vaccination to systemic administration of antisense oligonucleotides to local administration of mutant survivin adenoviruses. Initial clinical testing of one or more of these strategies might start in the not-too-distant future, and this will provide a more detailed blueprint for the translational applicability of the survivin pathway in cancer therapy.

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