

# PATHWAYS OF APOPTOTIC AND NON-APOPTOTIC DEATH IN TUMOUR CELLS

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Defects in cell-death pathways are hallmarks of cancer. Although resistance to apoptosis is closely linked to tumorigenesis, tumour cells can still be induced to die by non-apoptotic mechanisms, such as necrosis, senescence, autophagy and mitotic catastrophe. The molecular pathways that underlie these non-apoptotic responses remain unclear. Several apoptotic and non-apoptotic pathways of cell death have been defined in normal physiology and during tumorigenesis, and these could potentially be manipulated to develop new cancer therapies. The mitotic-checkpoint molecule survivin — the inactivation of which induces the death of p53-deficient cells by mitotic catastrophe — is of particular interest.

New genetic and biochemical approaches have fostered remarkable progress in our understanding of cancer biology during the past decade<sup>1</sup>. One of the most important advances has been the recognition that resistance to cell death — particularly apoptotic cell death — is an important aspect of both tumorigenesis and the development of resistance to anticancer drugs<sup>1–3</sup>. Much recent research on new cancer therapies has therefore focused on devising ways to overcome this resistance and to trigger the apoptosis of tumour cells. However, most tumour cells retain the ability to sustain permanent growth arrest or undergo non-apoptotic types of cell death, such as necrosis, autophagy, senescence and mitotic catastrophe<sup>4</sup>. It might therefore be possible to exploit these properties to induce tumour-cell death by non-apoptotic means and to confound the survival of drug-resistant clones. Importantly, the fraction of tumour cells that undergo non-apoptotic death increases if apoptosis is inhibited in response to anticancer agents or radiation<sup>5</sup>. A strategy that deliberately induces non-apoptotic cell death could therefore be a useful adjunct to standard cancer therapies and might make a significant contribution to a favourable treatment outcome.

The mechanisms that regulate non-apoptotic cell death remain much less clear than those that regulate

apoptosis<sup>6–8</sup>. In this review, we outline the various mechanisms that underlie apoptotic and non-apoptotic pathways of cell death in normal physiological conditions and during tumorigenesis (FIG. 1). We then examine how these pathways in general, and the mitotic-checkpoint molecule **survivin** in particular, might be manipulated in new modes of cancer treatment.

## Apoptotic pathways

The most common and well-defined form of programmed cell death (PCD) is apoptosis, which is a physiological 'cell-suicide' programme that is essential for embryonic development, immune-system function and the maintenance of tissue homeostasis in multicellular organisms<sup>9–11</sup>. Dysregulation of apoptosis has been implicated in numerous pathological conditions, including neurodegenerative diseases, autoimmunity and cancer. Apoptosis in mammalian cells is mediated by a family of cysteine proteases known as the **caspases**<sup>12</sup>. To keep the apoptotic programme under control, caspases are initially expressed in cells as inactive procaspase precursors. When initiator caspases — such as caspase-8 and caspase-9 — are activated by oligomerization, they cleave the precursor forms of effector caspases, such as caspase-3, caspase-6 and caspase-7 (REFS 13–15). Activated effector caspases in turn cleave a specific set of cellular

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## Summary

- Resistance to apoptosis is considered to be a hallmark of cancer cells. Defects in apoptosis underlie not only tumorigenesis, but also resistance to cancer treatments.
- Defects in non-apoptotic cell-death pathways — such as necrosis, autophagy and mitotic catastrophe — are also associated with tumorigenesis. The senescence ‘living-death’ programme — which involves the genes that encode p53, WAF1, INK4A and the retinoblastoma protein — is crucial for both tumour suppression and anticancer therapy.
- Malignant transformation can be induced by the dysregulation of oncogenes and tumour-suppressor genes that have secondary functions in the autophagic pathway. Inactivation of the autophagy gene beclin 1 induces malignancy in a mouse model.
- Defects in genes that are required for mitotic catastrophe can contribute to tumorigenesis. Uncontrolled activation of mitotic kinases and defects in mitotic checkpoints are important causes of centrosomal aberrations and genomic instability.
- Survivin is essential for mitotic progression, and inactivation of this protein induces death by mitotic catastrophe. Survivin might be an ideal target for cancer treatment, because cell death induced by survivin loss is independent of BCL2 and p53.
- The interactions between non-apoptotic and apoptotic cell-death pathways are not yet clear. Further investigation of non-apoptotic pathways might reveal new targets for the design of therapeutics that are aimed at inducing the non-apoptotic death of cancer cells.

substrates, resulting in the well-known constellation of biochemical and morphological changes that are associated with the apoptotic phenotype<sup>14</sup> (TABLE 1).

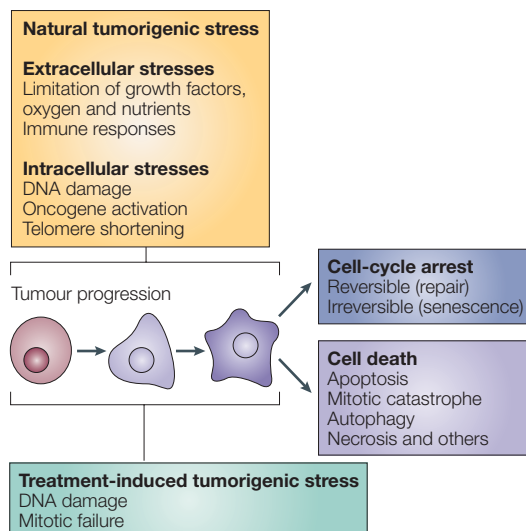
There are two pathways by which caspase activation is triggered — the extrinsic and intrinsic apoptotic pathways. The extrinsic pathway is activated by the engagement of death receptors on the cell surface. Binding of ligands such as FASL and tumour necrosis factor (TNF) to FAS and the TNF receptor (TNFR), respectively, induces the formation of the death-induced signalling complex (DISC). DISC in turn recruits caspase-8 and promotes the cascade of procaspase activation that follows<sup>16</sup>. The intrinsic pathway is triggered by various extracellular and intracellular stresses, such as growth-factor withdrawal, hypoxia, DNA damage and oncogene induction. Signals that are transduced in response to these stresses converge mainly on the mitochondria. A series of biochemical events is induced that results in the permeabilization of the outer mitochondrial membrane<sup>17</sup>, the release of cytochrome *c* and other proapoptotic molecules, the formation of the apoptosome — a large protein complex that contains cytochrome *c*, apoptotic protease activating factor 1 (APAF1) and caspase-9 — and caspase activation<sup>16</sup>. Among these processes, only the permeabilization step is regulated, in that anti-apoptotic members of the BCL2 family can stop the march towards apoptotic death<sup>18</sup>. Once cytochrome *c* is released, however, the downstream cascade of caspase activation is irreversible<sup>19,20</sup>.

Cell death is also modified by other mitochondrial proteins. Endonuclease G<sup>21</sup> and apoptosis-inducing factor (AIF)<sup>22</sup> might induce cell death independently of caspase activation. DIABLO (also known as SMAC)<sup>23,24</sup> and OMI (also known as HTRAZ)<sup>25–28</sup> promote caspase activation by counteracting inhibitor of apoptosis (IAP)-mediated caspase inhibition.

## Non-apoptotic pathways

The physiological roles and molecular pathways that are involved in non-apoptotic cell death are not fully understood, but caspases do not seem to be involved. Several apparently independent mechanisms have been identified, including necrosis, autophagy and mitotic catastrophe. Senescence can be considered to be a type of ‘living cell death’ because, although senescent cells maintain the integrity of their plasma membranes, they undergo permanent growth arrest and lose their clonogenicity. So, in this respect, defects in the senescence programme might contribute to tumorigenesis, making the induction of senescence a legitimate goal of cancer therapy. We have therefore included a discussion of senescence in this section.

**Senescence.** Cellular senescence was first described by Hayflick and Moorhead in 1961 (REF. 29). Primary cells in culture initially undergo a period of rapid proliferation, during which the telomeres of their chromosomes become significantly shorter. Eventually, cell growth decelerates and the cells enter a form of permanent cell-cycle arrest that is known as replicative senescence. A senescent cell typically shows morphological changes, such as a flattened cytoplasm and increased granularity. At the biochemical level, senescence is accompanied by changes in metabolism and the induction of senescence-associated  $\beta$ -galactosidase



**Figure 1 | Responses of cells to natural and treatment-induced tumorigenic stresses.** Both extracellular and intracellular stresses — generated by natural means or by treatment — can induce tumorigenic changes in normal cells. Cells then attempt to avoid transformation by undergoing either cell-cycle arrest or cell death. Cell-cycle arrest allows time for the repair of DNA damage. If repair is successful, the arrest is reversed and the cell divides. If the repair is unsuccessful, the cell survives, but becomes senescent and does not divide. If the repair is unsuccessful and the cell does not become senescent, cell death must be induced to prevent tumorigenesis. Several pathways, which involve some of the same signalling mediators, exist to carry out this function, including those that lead to apoptosis, mitotic catastrophe, autophagy and necrosis.

Table 1 | Characteristics of different types of cell death

Type of cell death	Morphological changes			Biochemical features	Common detection methods
	Nucleus	Cell membrane	Cytoplasm		
Apoptosis	Chromatin condensation; nuclear fragmentation; DNA laddering	Blebbing	Fragmentation (formation of apoptotic bodies)	Caspase-dependent	Electron microscopy; TUNEL staining; annexin staining; caspase-activity assays; DNA-fragmentation assays; detection of increased number of cells in subG1/G0; detection of changes in mitochondrial membrane potential
Autophagy	Partial chromatin condensation; no DNA laddering	Blebbing	Increased number of autophagic vesicles	Caspase-independent; increased lysosomal activity	Electron microscopy; protein-degradation assays; assays for marker-protein translocation to autophagic membranes; MDC staining
Mitotic catastrophe	Multiple micronuclei; nuclear fragmentation	–	–	Caspase-independent (at early stage) abnormal CDK1/cyclin B activation	Electron microscopy; assays for mitotic markers (MPM2); TUNEL staining
Necrosis	Clumping and random degradation of nuclear DNA	Swelling; rupture	Increased vacuolation; organelle degeneration; mitochondrial swelling	–	Electron microscopy; nuclear staining (usually negative); detection of inflammation and damage in surrounding tissues
Senescence	Distinct heterochromatic structure (senescence-associated heterochromatic foci)	–	Flattening and increased granularity	SA-β-gal activity	Electron microscopy; SA-β-gal staining; growth-arrest assays; assays for increased p53, INK4A and ARF levels (usually increased); assays for RB phosphorylation (usually hypophosphorylated); assays for metalloproteinase activity (usually upregulated)

CDK1, cyclin-dependent kinase 1; MDC, monodansylcadaverine; MPM2, mitotic phosphoprotein 2; SA-β-gal, senescence-associated β-galactosidase; RB, retinoblastoma protein.

activity<sup>30</sup>. At the genetic level, alterations to chromatin structure<sup>31</sup> and gene-expression patterns are seen. Senescence can also be induced by various cellular stresses<sup>32</sup>, and although cells in this situation do not show telomere shortening, they do show the other phenotypes that are characteristic of senescence.

Tumorigenic stresses such as DNA damage and oncogene activation, as well as agents that induce telomere shortening, can also trigger a cell to enter senescence. In this case, a senescence programme is initiated that induces the activation of various cell-cycle inhibitors and requires the functions of p53, the *CDKN1A* gene product WAF1 (also known as p21), the *CDKN2A* gene product INK4A (also known as p16), and the retinoblastoma protein (RB)<sup>33–36</sup>. The involvement of these tumour suppressors implies that one of the main functions of the senescence programme is to suppress tumorigenesis — a hypothesis that has been confirmed in animal models. For example, mutant mice carrying cells that are incapable of entering senescence develop cancer at an early age<sup>37–41</sup>. Conversely, the induction of premature senescence in murine mammary epithelial cells suppresses tumour development<sup>42</sup>.

**Necrosis.** In contrast to the self-contained nature of apoptotic cell death, necrosis is a ‘messy’, unregulated process of traumatic cell destruction, which is followed by the release of intracellular components. A distinctive

set of morphological features resulting from profound cellular damage is seen (TABLE 1), including membrane distortion, organelle degradation and cellular swelling<sup>9</sup>. Necrosis is usually a consequence of a pathophysiological condition, such as infection, inflammation or ischaemia. The resulting trauma causes extensive failure of normal physiological pathways that are essential for maintaining cellular homeostasis, such as regulation of ion transport, energy production and pH balance<sup>43</sup>. Although necrosis has been deemed to be a mainly passive process, the initiation and modulation of necrotic cell death are now under investigation at the molecular level. So far, however, the underlying signalling pathways remain a mystery.

**Autophagy.** One form of non-apoptotic, non-necrotic cell death is associated with a process that is known as autophagy. In normal cells, unwanted proteins or proteins that are no longer required are degraded by two independent mechanisms. One is the familiar ubiquitin-mediated proteolysis that takes place in proteasomes, and the other is autophagy — a mechanism by which long-lived proteins and organelle components are directed to and degraded within lysosomes<sup>44,45</sup>. Accumulating evidence has indicated that autophagy is conserved in various species and is activated in response to growth-factor withdrawal, differentiation and developmental triggers. Cells that undergo excessive autophagy are induced to die

in a non-apoptotic manner, and the morphology of autophagic cells is distinct from those that undergo either necrosis or apoptosis<sup>46,47</sup> (TABLE 1).

Although the morphology of autophagy was first described in mammalian cells, most of the known autophagy-related genes (ATGs) were discovered through genetic analyses in yeast<sup>48</sup>. Sixteen genes have been reported to function in the yeast autophagy pathway, many of which are conserved in other organisms<sup>49</sup>. In yeast, the principle function of autophagy is to sustain survival during nutrient starvation by catabolizing intracellular components and eliminating damaged organelles<sup>50</sup>. Following the induction of autophagy, autophagic vesicles or autophagosomes are formed through the assembly and expansion of double-layered, membrane-bound structures of unknown origin (in mammals, these are generally thought to originate in the endoplasmic reticulum) around whole organelles and isolated proteins. The autophagosome encapsulates the cytosolic materials, then docks and fuses with lysosomes or other vacuoles, causing degradation of the autophagosomal contents. At the molecular level, the signalling pathway that leads to autophagy involves at least the activities of phosphatidylinositol 3-kinase (PI3K) and the kinase target of rapamycin (TOR). Class-III PI3K activity is particularly important for the early stages of autophagic vesicle formation. By contrast, TOR negatively regulates autophagosome formation and expansion. Consequently, inhibition of TOR by rapamycin blocks cell-cycle progression<sup>51</sup> and eventually results in autophagy<sup>52</sup>. Deprivation of amino acids, nutrients or growth factors can also downregulate TOR signalling. The TOR pathway therefore coordinates signalling pathways that are initiated by nutritional and mitogenic factors, and also controls both protein synthesis and degradation.

Although the components of the autophagic machinery are highly conserved in a wide range of organisms<sup>53</sup>, the physiological role of the process itself varies. Genetic studies in *Caenorhabditis elegans* have shown that death by autophagy is required for embryonic development in this organism<sup>54</sup>, and developmental death by autophagy also occurs in *Drosophila*<sup>55</sup> and *Xenopus*<sup>56</sup>. However, the precise function of cell death by autophagy in mammals is not yet fully understood<sup>57,58</sup>. There is evidence that lysosomal degradation of organelles is required for cellular remodelling due to differentiation, stress or damage following exposure to cytotoxins. Other recent reports indicate that dysregulation of autophagy can result in pathological states such as neurodegenerative diseases<sup>59</sup>, cardiomyopathy<sup>60</sup>, myopathy<sup>61</sup>, infectious diseases and cancer<sup>62</sup>.

**Mitotic catastrophe.** The term mitotic catastrophe was originally coined by Paul Russell and Paul Nurse to describe the lethal fate of *Schizosaccharomyces pombe* cells that are forced to enter mitosis prematurely due to overexpression of Cdc2 (REF. 63). More recently, mitotic catastrophe has taken on a broader definition and has been used to explain the type of mammalian cell death

that is caused by aberrant mitosis. Mitotic catastrophe is associated with the formation of multinucleate, giant cells that contain uncondensed chromosomes, and is morphologically distinct from apoptosis, necrosis and autophagy (TABLE 1).

In normal somatic cells, the M phase of the cell cycle encompasses two processes: mitosis, in which sister chromatids are aligned and then segregated into two daughter cells; and cytokinesis, in which the cytoplasm and its contents are partitioned into those cells. Mitosis is further subdivided into prophase, prometaphase, metaphase, anaphase and telophase (BOX 1). Entry into M phase from G2 phase is driven by the activation of the CDK1 (the mammalian homologue of Cdc2)–cyclin complex, a process that is fairly well understood. Briefly, prior to mitosis, CDK1 is held in an inactive state by phosphorylation, which is mediated by the kinases WEE1 and MYT<sup>64</sup>. However, following activation by the phosphatase CDC25C, CDK1 phosphorylates a large number of substrates that promote nuclear-envelope breakdown, centrosome separation, spindle assembly and chromosome condensation<sup>65–67</sup>. Towards the end of normal mitosis, CDK1 is again phosphorylated and inactivated so that cytokinesis can proceed<sup>68</sup>.

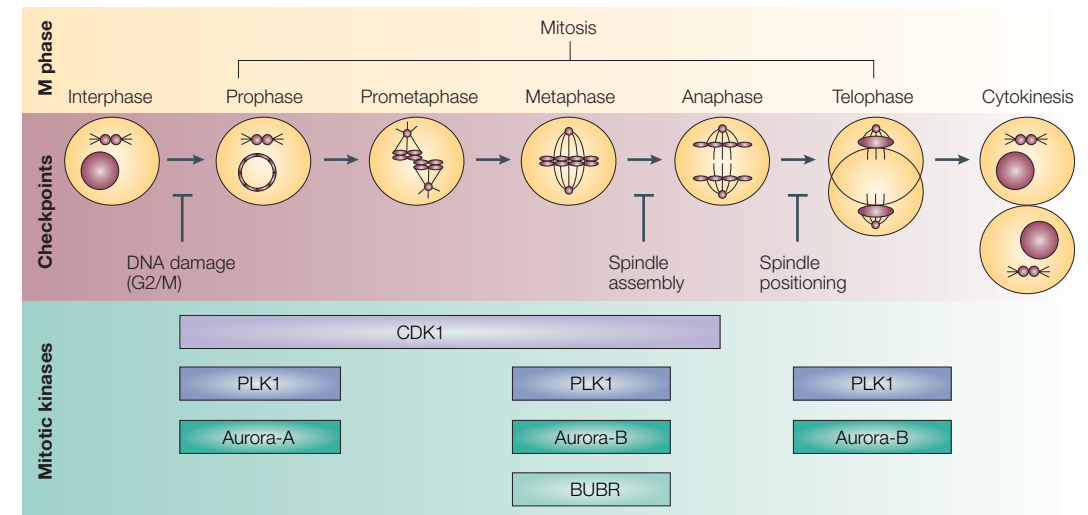
The G2 checkpoint of the cell cycle is responsible for blocking mitosis when a cell has sustained an insult to its DNA. DNA damage activates a number of molecules that promote cellular activities such as cell-cycle arrest, DNA repair or apoptosis, if the damage cannot be repaired. However, if the G2 checkpoint is defective, a cell can enter mitosis prematurely, before DNA replication is complete or DNA damage has been repaired. This aberrant mitosis causes the cell to undergo death by mitotic catastrophe.

Activation of the G2 checkpoint begins with the detection of DNA damage by the sensory molecules ataxia teleangiectasia mutated (ATM) and ATM- and Rad3-related (ATR). Activation of ATM and ATR in turn activates checkpoint kinase 2 (CHK2) and checkpoint kinase 1 (CHK1), respectively (BOX 1). These kinases phosphorylate CDC25C, the molecule that is responsible for activating CDK1. Phosphorylated CDC25C associates with the p53 target molecule 14-3-3 $\sigma$  and is exported from the nucleus, resulting in CDK1 inactivation and the establishment of G2 arrest<sup>69</sup>. p53 sustains G2 arrest through another downstream target molecule, WAF1 (REFS 70,71). WAF1 binds to CDK1–cyclin complexes and blocks their activation, and also inhibits proliferating-cell nuclear antigen (PCNA) activity<sup>72</sup>. The inhibition or inactivation of any of these G2-checkpoint genes — including those that encode ATR<sup>73,74</sup>, CHK1 (REF. 75), p53 (REF. 76), WAF1 (REF. 77) and 14-3-3 $\sigma$ <sup>71</sup> — results in the death of cells that have sustained DNA damage by mitotic catastrophe.

The transduction of genotoxic signalling to CHK1 and CHK2 is complex and involves many molecules that are associated with tumorigenesis. Histone H2AX, p53-binding protein 1 (53BP1), BRCA1 and Nijmegen breakage syndrome 1 (NBS1) are all targets of ATM and ATR. Phosphorylated H2AX recruits 53BP1, BRCA1, mediator of DNA damage checkpoint protein 1

Box 1 | **The M phase of the mammalian cell cycle.**

Mitosis occurs in the indicated phases to duplicate chromosomal DNA and ensure that both daughter cells receive the correct genetic complement. Cytokinesis then partitions the cytoplasmic components evenly between the daughter cells. The integrity of M-phase progression is monitored by three main checkpoints: one that detects DNA damage (G2/M arrest), one that detects mitotic-spindle malformations and one that detects incorrect positioning of the spindle. Defects in genes that are required for inducing mitotic catastrophe can contribute to tumorigenesis. These genes include those that encode the numerous kinases that are involved in mitotic regulation, such as polo-like kinase 1 (PLK1), the aurora kinase family (aurora-A and aurora-B), and a regulator of the spindle checkpoint called BUB-related kinase (BUBR). In response to genotoxic stress, ataxia teleangiectasia mutated (ATM) and ATM- and Rad3-related (ATR) become activated and, in turn, activate the checkpoint kinases CHK2 and CHK1, respectively. These kinases phosphorylate and inactivate CDC25, the molecule that is responsible for cyclin-dependent kinase 1 (CDK1) activation. When it is phosphorylated, CDC25 is translocated into the cytosol together with 14-3-3 $\sigma$ , and this blocks CDK1/cyclin activation, establishing G2 arrest.



(MDC1) and the MRE11–RAD50–NBS1 complex to the site of DNA damage. Accordingly, H2AX-deficient cells show a G2-checkpoint defect and genomic instability in response to ionizing radiation<sup>78</sup>. Similarly, 53BP1 binds to p53, CHK2, structural maintenance of chromosome 1 (SMC1) and BRCA1 at foci that are induced by DNA damage, and mediates downstream DNA-damage signalling. 53BP1-deficient cells also show a G2-checkpoint defect<sup>79–81</sup>, and 53BP1-deficient mice are cancer-prone<sup>82</sup>. BRCA1 is necessary for the activation of CHK1, which leads to DNA-damage-induced G2 arrest<sup>83</sup>, and MDC1 is important for the formation of DNA-damage-induced foci. Cells that lack MDC1 are sensitive to ionizing radiation and show a G2-checkpoint defect<sup>84–86</sup>.

Agents that damage microtubules and disrupt the mitotic spindle also cause mitotic catastrophe. For example, the drug paclitaxel induces an abnormal metaphase in which the sister chromatids fail to segregate properly<sup>87</sup>. CDK1 activation is prolonged abnormally in paclitaxel-treated cells, which undergo death by mitotic catastrophe.

Mitotic catastrophe seems to be a highly conserved stress-response mechanism. In *Drosophila*, Chk2 regulates mitotic catastrophe in a p53-independent manner<sup>88</sup>, and cells undergoing death by mitotic catastrophe have been seen both in patients with Alzheimer's disease<sup>89</sup> and in normal chick embryos<sup>90</sup>. Although the molecular mechanisms that regulate mitotic catastrophe

remain to be clarified, recent evidence has pointed to an important role for the cytoprotective molecule survivin. Studies of survivin-null mice and *in vitro* inactivation of survivin by RNA interference indicate that this molecule maintains the spindle checkpoint and prevents the accumulation of stressed cells that would otherwise undergo aberrant mitosis<sup>91–93</sup>.

**Cell-death defects and tumorigenesis**

**Defects in apoptosis.** Certain mutations allow a cell to resist death stimuli, and therefore provide it with a selective growth advantage. The cells of a developing tumour need to avoid apoptosis that is induced not only by unregulated oncogene expression<sup>94,95</sup>, but also by limited supplies of growth factors, oxygen or nutrients. Only by overcoming these threats to survival can the cancer cells go on to become invasive and metastatic, and capitalize on their ability to grow in new environments.

Studies of the anti-apoptotic gene *BCL2* and the *TP53* tumour-suppressor gene have highlighted the importance to tumour cells of overcoming apoptosis. In early studies of human B-cell follicular lymphomas, a chromosomal translocation linking the *BCL2* gene to an immunoglobulin locus was identified in the transformed cells. *BCL2* became constitutively active as a result of the translocation, driving the survival of the lymphoma cells<sup>96,97</sup>. Later studies

confirmed that the oncogenic activity of *BCL2* in this situation depends on its anti-apoptotic activity<sup>98–100</sup>. *BCL2* overexpression has also been shown to induce MYC-dependent lymphomagenesis *in vivo*. In this case, the proliferative function of MYC was unaffected, indicating that *BCL2* can counteract MYC-induced apoptosis<sup>99,101</sup>.

The tumour suppressor p53 is a well-known master regulator of apoptosis<sup>102–104</sup>. When p53 is functioning normally, genotoxic stress causing DNA damage that cannot be repaired during cell-cycle arrest induces apoptosis through the p53 pathway. Other cancer-related stresses, such as growth-factor withdrawal, hypoxia and dysregulated expression of mitogenic oncogenes can also induce p53-mediated apoptosis *in vitro*<sup>105</sup>. *In vivo*, p53-deficient mice show defects in apoptosis<sup>106,107</sup>, and p53 deficiency accelerates tumorigenesis in many settings.

There are also links between oncoproteins and components of the extrinsic and intrinsic apoptotic pathways that enhance tumorigenesis. For example, expression of the oncoprotein E1A synergistically enhances apoptosis induced by FAS, TNF or TRAIL<sup>108</sup>. Similarly, loss of APAF1 function seems to be important for the oncogenic transformation of melanoma and ovarian cancer cell lines<sup>109–111</sup>. The *MYC* oncogene has also been implicated in the FAS-induced death of several cell types<sup>112,113</sup>. As the FAS pathway regulates the immune system through its pro-apoptotic function, disruption of this pathway leads to lymphoproliferative disorders and haematopoietic cancers<sup>114</sup>. Somatic mutations of the *FAS* gene or its downstream effectors have been found in patients with multiple myeloma<sup>115</sup>, **non-Hodgkin's lymphoma**<sup>116</sup> and other cancers<sup>117,118</sup>.

Alterations to cell-survival pathways are also crucial for tumorigenesis because they can suppress or alter apoptosis. For example, the PI3K–**AKT** survival-signalling pathway is activated by various intracellular and extracellular stimuli. Once it is activated, the AKT kinase modulates transducers that function in apoptotic pathways, resulting in the ability of the cell to resist death signals. For example, AKT signalling induces the expression of the anti-apoptotic molecule BCL-X<sub>L</sub> and inhibits the pro-apoptotic activity of FKHL1 (also known as FOXO3A)<sup>119</sup>. In addition, AKT promotes the nuclear translocation of the E3 ubiquitin ligase **MDM2**, thereby negatively regulating p53-mediated apoptosis<sup>120</sup>. AKT activation also provides cells with a survival advantage through its promotion of glucose metabolism<sup>121,122</sup>. The PI3K–AKT survival pathway is itself subject to regulation, as it is activated by the RAS oncoprotein<sup>123</sup> and negatively regulated by the tumour suppressor PTEN. Another survival factor that is relevant to human tumorigenesis is nuclear factor-κB (NF-κB), a transcription factor that is activated by numerous cytokines and oncogenes. *De novo* gene transcription that is induced by NF-κB prevents apoptosis that is induced by the engagement of death receptors<sup>124–125</sup>. So, many different routes to the inactivation of pro-apoptotic signalling pathways underlie tumorigenesis.

**Defects in the induction of non-apoptotic cell death.** Defects in non-apoptotic cell-death pathways have also been linked to cancer. A substantial amount of evidence indicates that uncontrolled protein degradation by the proteasomal pathway can contribute to tumorigenesis. For example, signalling by WNT and hedgehog (HH) is regulated by the turnover of the β-catenin<sup>126</sup> and cubitus interruptus (CI)<sup>127</sup> proteins, respectively. Mutations in the genes that encode β-catenin and CI that constitutively activate the WNT and HH pathways are common in human colon cancer and basal-cell skin carcinomas, respectively<sup>128</sup>. Similarly, levels of p53 protein are negatively regulated by MDM2 (REF. 129–131). *MDM2* amplification — which leads to inadequate levels of p53 protein and a deficit in tumour suppression — has frequently been seen in soft-tissue sarcomas<sup>132</sup>. The ubiquitin-ligase complex that contains the von Hippel–Lindau (VHL) protein targets and inactivates the hypoxia-inducible transcription factor HIF1α<sup>133</sup>. HIF1α regulates the expression of vascular endothelial growth factor, erythropoietin and platelet-derived growth factor<sup>134</sup>. In the absence of VHL function, continuous activation of HIF1α promotes angiogenesis, which supports the growth of renal-cell carcinomas.

Defects in the autophagic pathway of protein degradation might also be connected to cancer. Some oncogenes and tumour-suppressor genes have been reported to have effects on this pathway. One aspect of malignant transformation induced by oncogenes and tumour suppressors could therefore be dysregulation of, or functional defects in, the autophagic pathway. For example, autophagy is partly controlled by the PI3K pathway<sup>135</sup>, and constitutive activation of PI3K signalling is common in human cancer cells. PI3K and its downstream effectors, AKT and TOR, might normally contribute to the suppression of autophagy<sup>136</sup>, whereas PTEN — a tumour suppressor that negatively regulates PI3K signalling — might normally promote autophagy<sup>137</sup>. It is possible that hyperactivation of the AKT pathway and TOR signalling, or loss of PTEN function, could inhibit both apoptotic and autophagic cell death, thereby contributing to cellular transformation by two means. The *RAS* and *MYC* oncogenes might also induce tumorigenesis partly by inhibiting autophagy, as the normal RAS pathway has been implicated in this form of cell death<sup>138</sup>, and overexpression of *MYC* in rat fibroblasts induces autophagy<sup>139</sup>.

Perhaps the best evidence that impaired autophagy is linked to tumorigenesis comes from studies of *BECN1*, the gene encoding **beclin 1**. This gene is the human homologue of the yeast autophagy gene *ATG6*, which is a component of the yeast PI3K complex<sup>140</sup>. In mammalian cells, *BECN1* interacts with PI3K and participates in the induction of autophagy in response to starvation<sup>141</sup>. *BECN1* has been mapped to a human tumour-susceptibility locus (17q21) and is monoallelically deleted in a high percentage of human **ovarian**, **breast** and **prostate** cancers<sup>142</sup>. *In vitro*, transfection of *BECN1* into a transformed cell line can decrease its tumorigenic potential<sup>62</sup>. Finally, studies of mice that are deficient for this protein have shown

Table 2 | Kinases involved in the regulation of mitosis

Kinase family	Mammalian family members	Localization	Functions
Aurora kinases	Aurora-A Aurora-B  Aurora-C	Spindle poles Kinetochore; midzone Centrosomes	Regulates centrosome function and spindle assembly Regulates chromosomal segregation, spindle orientation and microtubule dynamics Unknown
Polo kinases	PLK1  PLK2  PLK3	Centrosomes; kinetochores Kinetochores; mitotic spindle Midbody; centrosome; mitotic spindle; Golgi	Regulates mitotic entry and exit, and cytokinesis  Regulates mitotic entry and exit, and cytokinesis; downstream target of p53 Regulates mitotic dynamics and centrosomal function; involved in DNA-damage stress response and Golgi dynamics
CDKs	CDK1		Regulates mitotic entry and exit; associates with cyclin A and cyclin B
NIMA kinases	NEKs 1 to 11		NEK2 regulates centrosomal organization
Spindle-checkpoint kinases	BUB1; BUBR1; TTK; ESK	Kinetochore; centrosome (TTK and ESK only)	Regulate mitotic checkpoint to ensure accurate chromosomal segregation; activated by signals from unattached kinetochores to prevent premature entry into anaphase

BUB, budding uninhibited by benzimidazole; BUBR1, BUB-related kinase 1; CDK, cyclin-dependent kinase; ESK, EC STY kinase; PLK, polo-like kinase; NEK, NIMA-related kinase; NIMA, never in mitosis, gene A, kinase.

that BECN1-mediated regulation of autophagy is required for normal mammalian development, and that animals with heterozygous deletions in *Becn1* show a marked increase in the incidence of lymphomas and carcinomas of the lung and liver<sup>143,144</sup>. Deletion of mouse *Becn1* also results in hyperproliferative changes in mammary tissues<sup>143</sup>. Although these findings strongly point to the function of BECN1 in autophagy as the reason for its tumour-suppressive activity, it cannot be ruled out that another mechanism might be involved. BECN1 has been identified as a protein that binds to the anti-apoptotic molecule BCL2 (REF. 145). The mechanism and consequences of the BECN1–BCL2 interaction remain to be elucidated.

These studies indicate that autophagic degradation is an important means of preventing cellular transformation, and there are several mechanisms by which inhibition of this process might lead to tumour initiation. As autophagy is a form of PCD, reduced autophagic activity might simply increase survival. For example, without autophagy, the natural turnover of a protein that acts as a positive regulator of cell growth might be blocked, promoting proliferation. Autophagy is also involved in removing damaged organelles and, therefore, in maintaining cellular homeostasis. Damage to mitochondria or sections of the endoplasmic reticulum might result in the production of endogenous cellular oxidants that increase the basal mutation rate. Removal of these damaged internal cellular structures by autophagy might therefore limit the genotoxic damage that is caused by oxidants. Conversely, reduced autophagy might increase oxidant stress and promote the accumulation of tumorigenic mutations.

Defects in genes that are required for mitotic catastrophe can also contribute to tumorigenesis. These genes include those that encode the numerous

kinases that are involved in mitotic regulation, such as the polo-like kinase (PLK) family, the NIMA (for never in mitosis, gene A) family, the aurora kinase family and a regulator of the spindle checkpoint called BUB (TABLE 2). It is still unknown how defects that affect these enzymes induce aberrations in mitosis, as the exact downstream targets and regulatory mechanisms of these enzymes are not fully understood<sup>146</sup>. Evidence for a connection between loss of mitotic kinase function and mitotic catastrophe has come from several sources. First, a dominant-negative form of the **PLK1** enzyme, which is crucial for entry into mitosis, induces mitotic catastrophe<sup>147</sup>. Second, the gene that encodes the related kinase **PLK2**, which regulates mitotic progression, is a downstream target of p53. PLK function might therefore contribute to the maintenance of genetic stability<sup>148</sup>.

Another connection between mitotic catastrophe inhibition and cancer has come to light from studies of the aurora kinases. The *aurora-A* gene, which encodes a kinase, is located in the chromosomal region 20q13, which is amplified in a wide range of human cancers<sup>149,150</sup>. Overexpression of aurora-A increases genetic instability, aneuploidy and centrosomal aberrations *in vitro*. Significantly, loss of p53 enhances this phenotype. The overexpression of other mitotic kinases results in multinucleation and concomitant increases in the number of centrosomes<sup>151</sup>. Aurora-A is known to phosphorylate p53, which leads to its ubiquitylation by MDM2 and subsequent proteolysis. So, amplification of the *aurora-A* gene might result in its overexpression, which would lead to increased degradation of p53 and facilitate oncogenic transformation<sup>152</sup>. These findings indicate that uncontrolled activation of mitotic kinases and defects in mitotic checkpoints are important causes of the centrosomal aberrations that are so frequently seen in human tumour cells.

### Therapeutic induction of tumour-cell death

Basic and clinical studies have clearly established that most anticancer agents induce apoptosis, and that inhibition of apoptotic programmes can reduce the sensitivity of tumour cells to anticancer treatment. However, sensitivity of isolated tumour cells to apoptosis does not always correlate with sensitivity of the whole tumour to anticancer treatment. For example, *TP53* mutations are not always associated with reduced toxicity of anticancer agents, and loss of p53 can even increase cell death under some conditions<sup>153–155</sup>. Similarly, BCL2 overexpression does not increase cell viability following radiation or drug treatment in all settings. Indeed, BCL2 overexpression can simply delay drug- or radiation-induced apoptosis without significantly enhancing host survival<sup>156–159</sup>. These results indicate that the induction of apoptosis is not the whole answer in cancer therapy, and that non-apoptotic mechanisms are frequently responsible for treatment-induced tumour-cell death.

It is likely that apoptotic and non-apoptotic programmes contribute to cell death to varying extents in different cell types, and that the impact of each type of cell death varies depending on the mode of cancer treatment. Indeed, the concerted action of both apoptotic and non-apoptotic programmes is probably required to achieve a reduction in tumour size that minimizes the chance of relapse. This hypothesis has been confirmed in an elegant study of anticancer-drug responses in a transgenic mouse model of lymphomagenesis<sup>160</sup>. In this system, the acute response of lymphomas to anticancer agents is principally due to apoptosis. However, the remaining lymphoma cells then enter a senescence-like state that depends on the intact functions of p53 and INK4A. For anticancer therapy to be effective in these transgenic animals, both drug-induced apoptosis and senescence must occur. Moreover, neutralization of the lymphoma cells by drug-induced senescence is essential for the long-term survival of these mice, confirming that non-apoptotic forms of cell death are crucial for effective anticancer therapy.

Tumour cells that are treated with anticancer drugs also undergo death by autophagy. For example, breast carcinoma cells that are treated with the oestrogen-receptor antagonist tamoxifen accumulate autophagic vacuoles shortly before dying. Extensive autophagic degradation of the Golgi apparatus, polyribosomes and endoplasmic reticulum is seen. Morphologically, the dead cells lack the features of cells that have undergone apoptosis<sup>47,161</sup>. The mechanisms by which cancer cells select their mode of cell death in response to a given anticancer agent remain a mystery.

At present, few conventional anticancer treatments aim to induce the death of cancer cells by mitotic catastrophe. Studies have shown that targeting this pathway might represent a fresh approach to tumour elimination that does not depend in any way on apoptosis or autophagy. A key molecule that can be inactivated to induce mitotic catastrophe is survivin.

### Survivin regulates mitotic progression

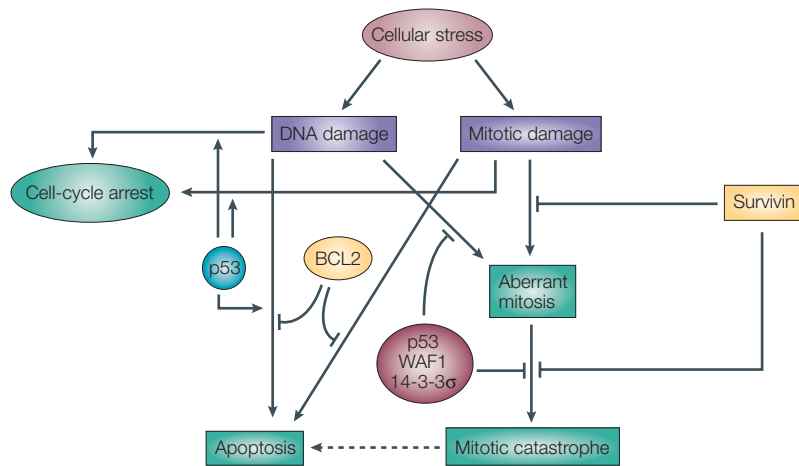
Survivin is a member of the IAP family. It is not highly expressed in most adult tissues, but high levels have been seen in fast-growing cells, such as transformed cell lines and human cancer cells. Deficiency for survivin induces cell death, and overexpression of survivin confers resistance to cell death. However, whether survivin is directly involved in the regulation of apoptosis remains controversial<sup>162–164</sup>. *In vitro*, survivin binds to caspases and inhibits their activation, just as other IAP family members do. In particular, survivin seems to regulate the localization and activation of caspase-3 during mitosis<sup>165</sup>. It has also been proposed that a splice variant of survivin might localize to mitochondria and regulate the intrinsic apoptotic pathway<sup>166</sup>, although this hypothesis awaits direct demonstration.

Other lines of evidence, however, argue against a role for survivin in the control of apoptosis. Structural analyses of survivin indicate that any effect that it might have on caspases must be indirect, as it lacks the amino-acid sequence that is essential in other IAPs for caspase binding. In addition, a cofactor that is not required by any other IAP is necessary for the inhibition of caspase activity by survivin<sup>167</sup>. Studies of cells from survivin-knockout mice have cast further doubt on the existence of a direct link between survivin and apoptosis. Survivin-deficient T cells show normal susceptibility to various external apoptotic stimuli and show no defects in spontaneous induction of apoptosis *in vitro*<sup>91,168</sup>.

Rather than controlling apoptosis, the primary function of survivin seems to be the regulation of mitotic progression. The survivin gene is highly conserved in a wide range of organisms, and all of its orthologues are involved in mitotic regulation<sup>169–171</sup>. Accordingly, the production of the survivin protein is generally regulated in a cell cycle-dependent manner<sup>172,173</sup>. Immunohistochemical analysis has shown that survivin associates with mitotic-spindle microtubules, centromeres and intracellular mid-bodies<sup>165,171</sup>. Other studies have shown that survivin is a chromosomal passenger protein that regulates chromosome segregation by interacting with other passenger proteins, inner centromere protein and the kinase **aurora-B**<sup>162,171</sup>. Inactivation of mammalian survivin — or its orthologues in lower organisms — results in cytokinesis abnormalities, particularly spindle defects<sup>169,170,174</sup>. In mammalian cells, survivin is necessary for the maintenance of the spindle checkpoint<sup>92,93</sup>. Developing thymocytes that lack functional survivin suffer proliferative defects that are characterized by aberrant spindle formation and severe defects in aurora-B localization, chromosome segregation and cytokinesis<sup>91</sup>. These cells then undergo PCD that does not result in an apoptotic morphology. The accumulated evidence therefore indicates that survivin does not directly regulate apoptosis, but is crucial for mitotic progression.

### Inducing mitotic catastrophe

Other mechanistic insights into survivin function have been drawn from the study of primary mouse embryonic fibroblasts (MEFs) that lack this protein.



**Figure 2 | Model for survivin-mediated protection against mitotic catastrophe.** Cellular stresses induce both DNA damage and defects in the mitotic machinery (mitotic damage). DNA damage can lead to cell-cycle arrest, apoptosis or aberrant mitosis. Mitotic damage can lead to cell-cycle arrest, apoptosis or mitotic catastrophe through aberrant mitosis. In situations in which p53 activity has induced apoptosis, BCL2 expression can rescue cells from death. p53 — and its target genes that encode WAF1 and 14-3-3σ — also help to prevent aberrant mitosis and mitotic catastrophe. The normal function of survivin is to maintain the integrity of the mitotic spindle and promote mitotic progression. Loss of survivin induces cell-cycle arrest and cell death by mitotic catastrophe in a manner that is independent of both p53 and BCL2. However, loss of survivin also induces p53 and WAF1 expression, perhaps indirectly triggering p53-dependent apoptosis (dashed line).

Primary MEFs carrying a conditional-deletion allele of the survivin gene (MEF<sup>lox/lox</sup>) were generated using CRE-LOXP-MEDIATED RECOMBINATION. As for survivin-deficient thymocytes, disorganized mitotic spindles were commonly seen in early-passage MEF<sup>lox/lox</sup> cells. Survivin-deficient MEFs were larger than their control counterparts and had multipolar spindles. Many mutant MEFs also had multiple micronuclei and uncondensed chromosomes. Indeed, the mutant cells could no longer proliferate, and underwent death that was morphologically consistent with mitotic catastrophe.

What is the signalling pathway that leads to the cell death that is induced by loss of survivin? Expression of p53 and its downstream target WAF1 is induced in the absence of survivin, but neither deficiency for p53 nor overexpression of BCL2 can rescue PCD that is induced by survivin loss<sup>91</sup>. Paradoxically, the cell-cycle arrest that is seen in survivin-deficient cells was partially rescued in mice that also lacked p53 or WAF1 function. In addition, PCD induced by survivin loss was significantly increased in a TP53-null or CDKN1A-null genetic background. Prior to their deaths, these cells showed highly abnormal spindle formation. It is possible that loss of survivin triggers the same p53-dependent signalling pathway that is seen in cells in which the mitotic spindles have been disrupted by microtubule-damaging agents<sup>175</sup>.

It is clear that p53 is dispensable for the spindle checkpoint itself. However, activation of the p53 target protein WAF1 is necessary for the G1-like arrest that halts the cycling of cells with abnormal spindles. Without p53 or WAF1, this G1-like arrest is compromised and the cells can ENDODUPLICATE their DNA and become polyploid<sup>176</sup>. Although the precise mechanism that

underlies the induction of p53 by spindle damage remains unknown, defects in centrosome duplication are able to activate the p53 pathway<sup>177</sup>. Furthermore, p53 can bind directly to aurora-A and suppress aurora-A-induced centrosome amplification<sup>178</sup>. In addition, WAF1 is highly upregulated in survivin-deficient cells, and loss of WAF1 releases the cell-cycle arrest that is induced by survivin loss. We therefore speculate that survivin is crucial for maintaining the integrity of spindles that have to form quickly in rapidly dividing cells. Consistent with this idea, inactivation of p53 sensitizes cells to PCD that is induced by agents that target microtubules<sup>154,155</sup>. It is not hard to imagine that loss of survivin coupled with p53 inactivation might have cumulative devastating effects on mitosis that could kill tumour cells (FIG. 2).

TP53 is mutated in more than 50% of human cancers. Because cell death induced by loss of survivin is independent of p53 and BCL2, it is feasible that survivin and/or molecules in the survivin pathway could be targeted for anticancer therapy. Targeted inactivation of survivin might induce mitotic catastrophe and cell death in tumour cells that have already lost p53 function and are therefore resistant to apoptosis. A drug that could specifically inactivate survivin in this way might be a significant addition to the anticancer-drug armamentarium.

**Future directions**

Tumorigenesis is a dynamic process that is driven by complex interactions between oncogene activation, tumour-suppressor inactivation and responses to cellular stress. How do cancer cells that are subjected to selective pressure or anticancer agents choose their fate from options such as apoptosis, cell-cycle arrest, necrosis, autophagy and mitotic catastrophe? Cancer cells often have a defect in a particular cell-death pathway, but the cells can still die (at least in theory) because of the redundancy of cell-death mechanisms. However, the nature of the cell-death defect ultimately affects the clinical outcome of treatment, depending on which mechanism is missing. Several questions remain concerning the interactions between apoptotic and non-apoptotic cell-death pathways. Are apoptosis, senescence, necrosis, autophagy and mitotic catastrophe entirely independent programmes, or do they overlap to some degree? Do these responses occur successively or simultaneously? Can one mechanism compensate for another that is inactivated by a tumorigenic mutation? If these cell-death pathways overlap significantly and are commonly modified in tumour progression, anticancer therapies that are designed to restore function to a key programme will restore them all, and therefore greatly improve the efficacy of the treatment. However, if these cell-death mechanisms are independent, identifying and targeting only the pathway that most efficiently inactivates the cancer cells in question might be the most effective approach.

Future investigations of the molecular mechanisms of non-apoptotic cell death and the interdependency of these pathways will yield fresh insights into the evolution of cell-death programmes, their roles in physiological cell death, and their potential for manipulation by new anticancer therapeutics.

**CRE-LOXP RECOMBINATION**  
A site-specific recombination system derived from bacteriophage P1. By flanking a gene of interest with loxP sites and expressing CRE recombinase under the control of an appropriate promoter, genomic manipulations such as deletion, integration, inversion or translocation of a gene of interest can be carried out in a tissue- or stage-specific manner.

**ENDODUPLICATION**  
A modified cell cycle in which S phase (DNA synthesis) occurs without M phase (mitosis and cell division). Large, polyploid cells result.

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#### Competing interests statement

The authors declare no competing financial interests.

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