

Cell proliferation and apoptosis

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Cell proliferation and cell death are essential yet opposing cellular processes. Crosstalk between these processes promotes a balance between proliferation and death, and it limits the growth and survival of cells with oncogenic mutations. New insights into the mechanisms by which strong signals to proliferate and activation of cyclin-dependent kinases promote apoptosis have recently been published, and a novel cell cycle regulated caspase inhibitor, Survivin, has been described.

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Abbreviations

ARF	alternative reading frame
ASK1	apoptosis signal regulating kinase 1
BIR	baculovirus IAP repeat
CDK	cyclin-dependent kinase
IAP	inhibitor of apoptosis
Rb	retinoblastoma

Introduction

A coordination and balance between cell proliferation and apoptosis is crucial for normal development and tissue-size homeostasis in the adult. Cancer results when clones of mutated cells survive and proliferate inappropriately, disrupting this balance. One mechanism for maintaining size homeostasis is a requirement for factor-dependent signaling from the environment for cell survival [1]. Cells that exhaust local supplies of these factors or that move to new locations away from the source will die. Although this mechanism of growth control is certainly part of the story, it is not sufficient to limit the expansion of clones that have a proliferation- or survival-promoting mutation. This is because either kind of mutation would be expected to cause an increase in the number of mutant cells relative to their normal neighbors.

A number of observations suggest that signaling between the proliferation and cell death machinery occurs: these include the observation that mutations that promote inappropriate entry into the cell cycle often also promote apoptosis (discussed below), and that overexpression of anti-apoptotic members of the bcl-2 family of proteins can suppress proliferation and promote entry into G₀ [2]. Second-site mutations that block death associated with inappropriate proliferation or that promote proliferation in the face of ectopic bcl-2 expression, dramatically promote

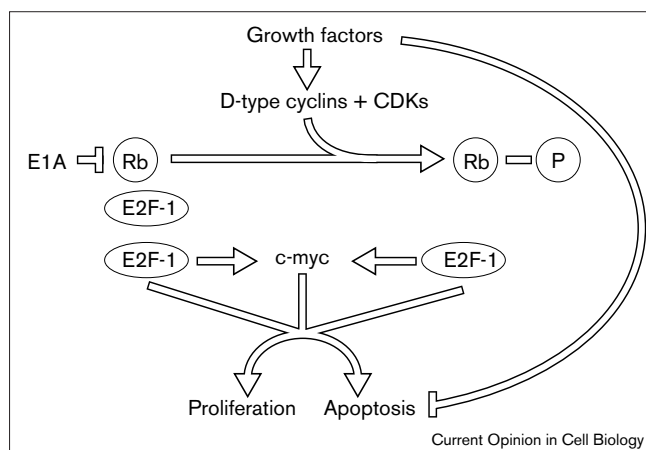
cancer development [3]. These observations suggest that an important function of communication between the proliferation and death pathways is to prevent the survival and expansion of clones of aberrant cells. Here we review several recent developments in our understanding of how signals associated with proliferation interface with the cell death machinery. We also discuss a new cell death regulator the IAP (inhibitor of apoptosis) family caspase inhibitor known as Survivin and discuss potential roles it might play.

Oncogene-induced apoptosis

A number of oncogenic mutations drive the cell to proliferate. Examples include mutations that cause overexpression of genes encoding cyclin D1, members of the myc family, adenovirus E1A, the G₁ transcription factor E2F-1, as well as loss of function mutations in the G₁ checkpoint retinoblastoma gene *RBI*. However, deregulation of these genes also often results in induction of apoptosis and increased sensitivity to a variety of apoptosis inducing agents [3]. These findings, in conjunction with observations showing that the presence of growth factors can prevent oncogene-induced death and that growth factor removal potentiates such death, have suggested a ‘dual signal’ model [3,4]. This model proposes that a proliferation stimulus leads to activation of a death signal, either directly as a consequence of entry into the cell cycle or through activation of a parallel pathway, and that successful proliferation can only occur if the apoptotic program is suppressed.

Although it is clear that a strong, oncogenic stimulus to proliferate is associated with death signaling, an important unresolved question is whether normal cell proliferation results in the production of a death stimulus that is qualitatively similar. If so, how is it that cells do not die during normal proliferation? There are several possibilities. Control of entry into the cell cycle by D-type cyclins and their cyclin-dependent kinase (CDK) partners may be one important regulatory mechanism. In normal cells, activation of molecules that drive cells into S phase, such as E2F and c-myc, occurs downstream of cyclin D–CDK-dependent phosphorylation of Rb family members. The increase in cyclin D levels, which drives this process, occurs as a consequence of growth factor signals from the environment that increase both the synthesis of cyclin D and its assembly with catalytic CDK partners, CDK4 and CDK6 [5]. Signal transduction by these same growth factors also acts to prevent apoptosis in a number of ways [6]. In contrast, when the expression of genes such as those encoding cyclin D itself, c-myc, E2F or adenovirus E1A (which leads to activation of E2F) is uncoupled from the presence of growth factors, cell cycle entry and associated death signaling may occur without concomitant anti-apoptotic signaling, resulting in cell death (Figure 1). It may also be that the levels of survival signaling are not normally limiting for cells undergoing normal proliferation but

Figure 1



Coordination of proliferation with anti-apoptotic signals. It is not clear whether the events of normal cell proliferation result in the production of a death signal similar to that arising from oncogene expression. Nonetheless, the available information provides a framework within which such an activity could be regulated to allow successful proliferation. Growth factor signaling results in the activation of receptor tyrosine and serine/threonine protein kinases (not shown). Many of these lead to activation of Ras, which signals through several different pathways (not shown) to stabilize and promote the synthesis of D-type cyclins, and their assembly with CDK4 or CDK6 [6]. Cyclin D-CDK complexes sequester CDK inhibitors associated with cyclin E-CDK2 (not shown) and also phosphorylate Rb. These events set up a positive-feedback cycle leading to further cyclin E-CDK2-dependent phosphorylation of Rb. Phosphorylation of Rb results in its release of members of the E2F family of transcription factors. These then activate genes required for entry into S phase, including *c-MYC* [21]. Expression of the adenovirus E1A gene also leads to dissociation of Rb-E2F complexes [21]. Expression of high, oncogenic, levels of the genes encoding the E2F family member E2F-1 or *c-myc* results in the production of a death signal in addition to promoting cell proliferation. In this model, it is hypothesized that normal cells produce a similar signal but that proliferation occurs because this death signal is inhibited as a result of growth factor signaling, acting in particular through the protein kinase Akt [6]. Also, as discussed in the Note added in proof, one mechanism by which *c-myc* overexpression promotes proliferation is by upregulating levels of cyclin D1 and cyclin D2.

that the continuous proliferation stimulus associated with oncogene expression simply leads to a much higher level of death signaling.

As discussed in detail below, oncogene-expressing cells produce death signals and are more sensitive than their normal counterparts to treatments that result in cellular damage and apoptotic signaling. This increased sensitivity probably provides one explanation for why radiation and chemotherapy, which induce apoptotic signaling by damaging DNA and disrupting the cell cycle, work well on primary tumors. The likelihood that tumor cells will be resistant to these treatments, which can lead to cancer progression, increases greatly, however, when other mutations occur that block steps in death signaling or execution. Bypassing these blocks and reactivating death signaling pathways specifically in tumor cells requires an understanding of how oncogenic proliferation signals lead to

activation of the death machinery, and the relationship these signals have to any that are produced during normal cell proliferation. One important conceptual advance has come from the finding that, at least in some cases, oncogene expressing cells that are resistant to chemotherapy are able to initiate activation of components of the apoptosis machinery. Thus the machinery can remain activatable, but somehow inhibited. Other recent insights into proliferation-associated death signaling and effector mechanisms are discussed below.

The p19^{ARF}-p53 pathway

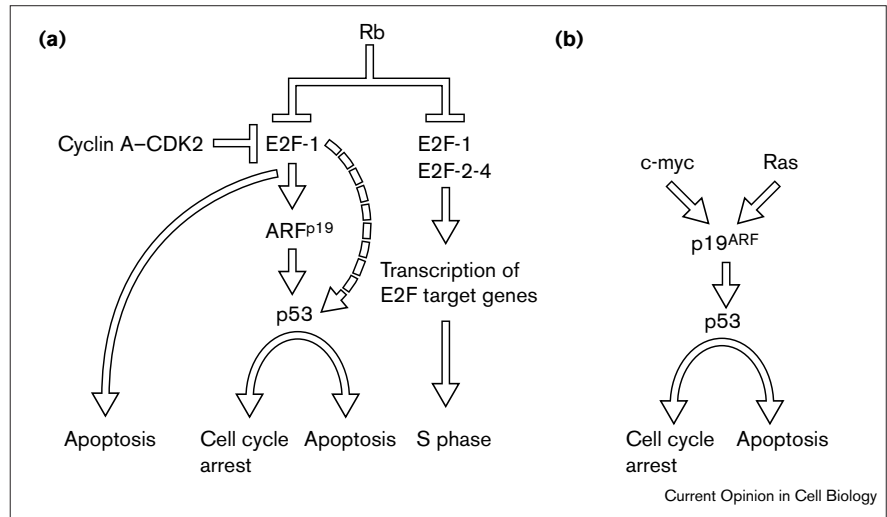
One of the most important links between the proliferation and cell death machinery is the tumor suppressor p53, which promotes cell cycle arrest or apoptosis in response to DNA damage or a strong oncogenic stimulus to proliferate [7]. Recent evidence shows that oncogene expression and DNA damage use different mechanisms to induce p53-dependent apoptosis [8]. The importance of the oncogene-dependent p53 death pathway is illustrated by the fact that most tumors have disruption of either p53 or an upstream activator of this pathway, the p19^{ARF} tumor suppressor. Expression of oncogenes such as those encoding E2F-1, *c-myc*, E1A or oncogenic versions of Ras result in the accumulation of p19^{ARF} [9[•],10[•],11^{••},12^{••}], the product of an alternative reading frame (ARF) from the *INK4A/ARF* locus [13]. This induction occurs through multiple mechanisms [9[•],12^{••}], and p19^{ARF} acts in several different ways to promote p53 stabilization and function [14–17]. Oncogene expression in *p19^{ARF}-/-* cells results in a strongly attenuated induction of p53 (though it is not lost completely) and these cells do not undergo apoptosis [11^{••},12^{••}]. Also, normal cells that survive senescence crisis or *c-MYC* overexpression generally show mutations in either *p53* or *p19^{ARF}* but not both, consistent with the idea that these genes function in the same death promoting pathway [12^{••},18]. Furthermore, *p19^{ARF}-/-* mice, like those lacking p53, are highly prone to tumor development [18]. Importantly, however, *p19^{ARF}-/-* cells show a normal induction of p53 following exposure to DNA damaging agents [18]. Also, cells lacking elements of the DNA-damage-dependent p53 pathway are normal with respect to oncogene-dependent p53 induction [19[•]]. Thus DNA-damage and oncogene expression induce p53 through separate pathways. p53 promotes apoptosis downstream of DNA damage or an oncogenic proliferation stimulus through a number of mechanisms (reviewed in [20]).

E2F-1: oncogene and tumor suppressor in the retinoblastoma pathway

Loss of Rb or deregulation of upstream regulators of Rb occurs in essentially all human tumors. One consequence of this deregulation is derepression of E2F family members, resulting in the transcription of genes required for S phase [21]. Loss of function of Rb is thus associated with increased proliferation; however, cell death is also prominent in Rb mutants [22]. Recent evidence demonstrates that E2F-1 plays an important role to limit the

Figure 2

The ARF–p53 pathway. (a) Loss of function of Rb leads to induction of E2F family members, including E2F-1. E2F family proteins transcriptionally activate genes required for entry into S phase. E2F-1 also transcriptionally activates the p19^{ARF} gene and promotes p19^{ARF} stabilization. p19^{ARF} acts in several ways to stabilize p53 [8,14–17], leading to either p53-dependent cell cycle arrest or apoptosis. High level expression of E2F-1 or c-myc also leads to p19^{ARF}-independent induction of p53, albeit this is an attenuated response. E2F-1 can also promote p53-independent apoptosis. Cyclin A–CDK2 phosphorylation of E2F-1 during S phase blocks its ability to bind DNA, thus inhibiting its activity [21]. Disruption of cyclin A–CDK2–E2F-1 interactions thus leads to increased E2F-1 activity, which may promote the death of cells already expressing high levels of the E2F-1 gene [25*]. (b) Expression of the genes encoding c-myc and Ras results in accumulation of p19^{ARF}, but there is no



evidence that this involves upregulation of E2F-1, suggesting that other mechanisms of p19^{ARF} induction exist. The fact that c-myc

and Ras can also promote proliferation is not shown.

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oncogenic consequences of Rb loss by promoting apoptosis through activation of several pathways, including p19^{ARF}–p53 (Figure 2). *E2F-1* is a potential oncogene because overexpression can drive entry into S phase and overcome G₁ arrest induced by inhibition of G₁ CDK activity or irradiation. However, in addition to promoting cell cycle activation, E2F-1, but not other E2F family members, can promote apoptosis through several different mechanisms [21]. These observations suggest that E2F-1 might function as a tumor suppressor, acting to signal cell death downstream of the loss of Rb function. Evidence for such a role comes from observations showing that E2F-1-deficient mice are predisposed to tumor formation [23], and that loss of E2F-1 can suppress apoptosis occurring in cells that have compromised Rb function [24**]. On the basis of these observations one might expect that loss of E2F-1 would promote cell growth. However, at least in the choroid plexus this is not the case because E2F-1 is also required for effective tumor cell proliferation [24**].

An important question for the future is to determine if E2F-1 is required for the proliferation of other tumor types. Because loss of Rb function occurs in many cancers, therapies aimed at potentiating E2F-1's ability to kill seem appropriate. One approach toward this end utilizes oligopeptides that contain a docking-site motif for cyclin–CDK complexes. Normally, S-phase phosphorylation of E2Fs by cyclin A–CDK2 causes the E2Fs to dissociate from DNA, thus reducing their activity. Peptides with the docking-site motif decrease the ability of cyclin–CDKs to phosphorylate substrates, including E2F-1, resulting in increased E2F-1 activity. In cells with compromised Rb function, which already have elevated

levels of E2F-1, increasing E2F-1 activity further tips these cells — but not normal cells which have much lower levels of E2F-1 — towards apoptosis [25*]. In other contexts, described below, in which E2F-1 levels are unlikely to be high, blocking CDK activity suppresses apoptosis.

Linking oncogene activation to the cell death machine

Most if not all apoptotic cell death signaling pathways ultimately lead to the activation of members of the caspase family of proteases, which act as signal transducers and death effectors [26]. There are two well-described pathways by which caspases that function as signal transducers (known as apical or upstream caspases) become activated. In one pathway initiating at the plasma membrane, ligand binding to a death receptor (CD95 being one example) results in recruitment of procaspase-8 into a multiprotein complex in which caspase autoactivation and transactivation occurs [27]. In a second pathway, cellular stress of various sorts causes the release of mitochondrial cytochrome c. This, in association with a cytoplasmic protein known as Apaf-1, recruits caspase-9 and leads to caspase-9 activation [28,29]. Apical caspases cleave downstream, or executioner caspases. Executioner caspases are thought to have a major role in cleaving cellular substrates that lead to cell death.

Several sets of observations point towards an important role for the cytochrome c, Apaf-1, caspase-9 pathway in mediating oncogene-dependent death signaling. Mouse embryo fibroblasts undergo p53-dependent apoptosis in response to c-MYC overexpression. Death, but not p53 induction, is blocked in versions of these cells in which the genes encoding Apaf-1 or caspase-9 have been knocked

out, consistent with the idea that these genes act downstream of p53 to promote death [30**]. Overexpression experiments using a dominant-negative caspase-9 as well as biochemical purification also indicate that Apaf-1 and caspase-9 are required for the death of cells expressing adenovirus *E1A* [31,32**].

It is likely that oncogene expression can act at several different points to regulate activation of this pathway. Thus, biochemical fractionation of cells expressing E1A shows that Apaf-1 and caspase-9 in these cells, but not normal cells, are somehow primed to be able to activate downstream caspases, but held inactive [31,32**]. The nature of the priming and inhibitory factors is unknown; however, the priming stimulus is unlikely to require p53 or release of cytochrome c because similar caspase activating activity is seen in extracts of cells expressing E1A and the adenovirus E1B genes [31], which inhibit a number of apoptotic pathways including those dependent on p53 or bax-mediated release of mitochondrial cytochrome c [33].

Control of the release of cytochrome c defines another major site of death regulation by oncogenes. Thus in Rat-1 cells overexpressing *c-MYC*, cytochrome c release occurs very early and does not seem to require caspase activation or the activation of other death pathways such as p53 or CD95. Cytoplasmic cytochrome c in these cells does not kill, but rather makes the cell sensitive to death stimuli transduced by other pathways [34**]. In these cells death is independent of p53 but instead requires the presence of an autocrine CD95 signaling loop [35]. In contrast, *E1A* expression does not result in cytochrome c release directly. Instead *E1A* expression seems to sensitize cells to the effects of other death stimuli that then lead to cytochrome c release [32**]. How *c-myc* and *E1A* regulate cytochrome c release is not known but the fact that a major target for oncogene-dependent death signaling is release of cytochrome c helps to explain why oncogene-dependent cell death can often be prevented by growth factor signaling pathways that lead to the activation of the serine/threonine kinase Akt, or by expression of anti-apoptotic *bcl-2* family members, both of which have control of mitochondrial release of cytochrome c as a focus of action [2,6,34**,36].

CDKs and CDK inhibitors: engines of the cell cycle, ... and cell death?

p53-dependent cell cycle arrest occurs as a consequence of induction of the CDK inhibitor p21^{CIP1} (p21). p21 acts at several points — during G₁, and at the G₂/M phase transition — to block cell cycle progression. It does this by inhibiting cyclin-CDK activity, and by inhibiting DNA replication but not DNA repair as a consequence of interactions with the proliferating cell nuclear antigen [37]. However, p21 also seems to have an anti-apoptotic function downstream of p53 induction because a decrease or loss of p21 gene expression in cells that would normally undergo cell cycle arrest leads instead to apoptosis [38,39]. Thus, the relative levels of p21 and p53-dependent death signals may

determine whether cells undergo p53-dependent arrest or death. As described below, caspase cleavage of p21 provides one mechanism by which this balance may be tipped towards death. A number of other observations also point to a role for p21 expression in preventing apoptosis [40,41,42**,43**,44–47], and suggest that p21 may suppress apoptosis in several different ways. Thus p21-dependent cell cycle withdrawal can lead to upregulation of survival signaling molecules such as Akt [45], and cytoplasmic p21 can interact with caspases [47] and the apoptosis signal regulating kinase 1 (ASK1) [44]. In addition, p21 appears to play an important role as an apoptosis inhibitor by virtue of its ability to perform one of the jobs it carries out in promoting cell cycle arrest — inhibiting CDK activity.

Evidence that CDK activity is important for promoting apoptosis comes from a number of observations showing that CDC2 and/or CDK2 — or in the case of neurons CDK4 and CDK6 — are upregulated in a number of cells undergoing apoptosis, that manipulations that decrease this activity prevent death, and that CDK upregulation promotes death [41,42**,43**,48–52,53*,54,55,56*,57]. However, not all apoptotic deaths require CDK activity [53*,58], and there is no evidence implicating them in the control of cell death in model organisms such as *Drosophila* or *Caenorhabditis elegans*. How does CDK activity, which normally promotes cell cycle progression, contribute to apoptosis? The answers are not in, but it seems unlikely that death occurs as a consequence of CDK-dependent induction of an aberrant cell cycle. This is because events typical of cell cycle progression downstream of CDK activation such as activation of polo-like kinase-1, entry into S phase or spindle formation do not occur [53*,56*].

How is CDC/CDK activity upregulated? A number of observations, with some exceptions [57], argue that CDK activation occurs downstream of death signal initiation and as a consequence of degradation or caspase-mediated cleavage of negative regulators of CDKs. Thus, cleavage of the CDK inhibitors p21 or p27^{KIP1} has been observed in a number of situations in which apoptosis occurs [43**,59,60], and overexpression of normal or uncleavable versions of these proteins provides strong protection against death [43**,60]. Degradation of p27^{KIP1} has been observed in other cells [53*]. Finally, cleavage of the CDC2 negative regulatory kinase *wee1*, which might be expected to compromise its ability to inhibit CDK activity through phosphorylation, has also been documented [56*].

There are several ways in which CDK activation downstream of caspase activation might contribute to caspase-dependent cell death. CDK phosphorylation might function as part of a positive-feedback loop that leads to further caspase activation by decreasing the activity of caspase inhibitors, such as the IAPs, or increasing the activity of other pro-apoptotic molecules. Alternatively, ectopic CDK activity might also lead to induction of other death pathways. The important point is that initiation of a

caspase cascade, particularly in the presence of caspase inhibitors, might not always be sufficient to sustain an apoptotic response. Activation of CDKs could provide a way of maintaining or amplifying this activity. Such amplification might be required for apoptosis in some contexts but not others, explaining why a requirement for CDK activation during apoptosis is not universal.

Presumably, the key to understanding the roles CDK activation plays in apoptosis will come from identifying the substrates phosphorylated. One possibility is that these substrates are proteins that are modified in similar ways in apoptotic and mitotic cells. These might include proteins contributing to the morphological similarities between apoptotic and mitotic cells, such as cell rounding, nuclear membrane breakdown or chromatin condensation. Alternatively, CDK activation during apoptosis might result in phosphorylation of death-specific targets. If this is correct an important question becomes how CDK activity is targeted to these substrates only during apoptosis? One way to achieve this might involve association of the kinase with novel cyclin partners. Consistent with this possibility, CDK2 activity in apoptosing thymocytes does not appear to be associated with its normal partners, cyclins A or E, and CDK2 activation requires protein synthesis [53•]. However, the identity of the postulated cyclin partners is unknown, and in other situations apoptosis-associated CDC or CDK activity does occur in association with normal cyclin partners [43•,51,57].

Survivin the cell cycle?

Some oncogene-expressing cells can survive the mitochondrial dysfunction associated with cytochrome c release if subsequent caspase activation is blocked [30••]. Although the possibility that these cells have acquired second site mutations that prevent cytochrome c release has not been ruled out, these results suggest that a mechanism by which transformed cells could escape death would involve upregulation of caspase inhibitors. Three mammalian IAPs, XIAP, cIAP1 and cIAP2 are expressed broadly in development and in the adult. Several of these are induced by Rel or NF- κ B [61], which function as oncoproteins in a number of contexts [62]. However, overexpression of these IAPs in actual tumors has not been documented. A recently identified human protein, Survivin [63] (TIAP in the mouse [64•]) is strikingly different. In contrast to the other described IAPs, Survivin shows prominent regulation at the transcriptional level. It is abundant during development in proliferating tissues and in tissues in which apoptosis is prominent, but is low or absent in terminally differentiated adult tissues [63,64•,65]. Most importantly, Survivin is upregulated in a number of common cancers and transformed cell lines [61,63,66•]. Additional evidence suggesting that Survivin may play an important anti-apoptotic role in cell proliferation and cancer progression comes from the findings that Survivin is upregulated in G₂/M [64•,67••] and that it is associated with spindle microtubules and seems to require this association for

anti-apoptotic activity at least with respect to the apoptosis inducer taxol [67••]. Also, overexpression of the gene encoding Survivin blocks cell death in response to a number of different stimuli [63,64•,66•,67••] and Survivin binds, although not well, to processed forms of caspase-3 and caspase-7 [64•,66•]. Finally, antisense Survivin promotes caspase activation and cell death, at least in HeLa cells [68].

One possibility suggested by these observations is that caspase activity occurs during each cell cycle and that Survivin functions to block this activity. This caspase activity might be required for some aspect of cell division. Alternatively, caspase activity and Survivin could act together as part of a G₂/M checkpoint. In such a model Survivin associated with microtubules might be required to block caspase activity during G₂/M. Disruption of microtubules would then lead to loss of Survivin function and an increase in death-promoting caspase activity. Survivin's functions might, however, be more complex than simple caspase inhibition. The worm *C. elegans* and the yeasts *S. cerevisiae* and *S. pombe* also have genes that encode small BIR-containing proteins (BIRPs), which have significant homology to Survivin, and these proteins are required for cytokinesis [69•,70•].

There are a number of unanswered questions. Is caspase activation in fact a normal part of movement through the cell cycle? Regardless of whether it is or not, what is the source of caspase activity found in cells with decreased Survivin function? Does it arise as a consequence of the loss of Survivin the caspase inhibitor, or as a secondary consequence of loss of a Survivin-dependent function that leads to caspase activation? Analysis of Survivin knockouts should be very illuminating. In particular it will be important to determine if cells that lack Survivin are able to progress through the cell cycle if caspase activity is blocked using other caspase inhibitors. If Survivin function as a caspase inhibitor does in fact serve as a downstream roadblock on the way to tumor cell apoptosis, the fact that it is essentially absent in normal postmitotic tissues in the adult makes it an exciting potential therapeutic target. Identifying molecules that can disrupt IAP-caspase interactions then provides one route to inducing tumor cell death [71••].

Conclusions

Tissue size homeostasis requires a balance between proliferation and cell death. An oncogenic stimulus to proliferate has the potential to disrupt this balance; however, this potential is kept in check because a strong proliferation stimulus also leads to the production of death signals that make these cells more sensitive to environmental conditions such as growth-factor deprivation or hypoxia. The E2F-1-p19^{ARF}-p53 pathway constitutes a vital mechanism by which oncogene-induced death signals are transduced, but other E2F-1-, ARF-, and p53-independent pathways exist as well. Activation of caspase-9 downstream of mitochondrial release

of cytochrome c is an important pathway by which oncogene-dependent death is carried out but the ways in which this pathway becomes activated are still largely unknown. Further downstream, caspase-dependent and independent activation of CDKs contributes to apoptosis in many cell types but the functions and relationship of this activity to that required for cell cycle progression are unknown. Does normal cell proliferation result in the production of a death stimulus that is qualitatively similar to that induced by oncogene expression? The identification of Survivin as a cell cycle-regulated caspase inhibitor also upregulated in tumors suggests that caspase activity may occur normally during phases of the cell cycle. But whether this putative caspase activity functions as a part of a proliferation checkpoint or has other roles is unknown, as are the normal roles of Survivin.

Note added in proof

Two recent reports [72^{**},73^{**}] demonstrate that an important mechanism by which c-myc induces proliferation involves upregulation of cyclins D1 and D2. Cyclin D-CDK complexes sequester the CDK inhibitors p21 and p27KIP1, thus promoting cyclin E-CDK activation. Interestingly, although cyclin D1 and D2 are required for myc-dependent proliferation, they are not required for myc-dependent apoptosis. Thus, these papers show that myc-dependent proliferation and apoptosis signaling are genetically separable. Cells from cyclin D^{-/-} mice that overexpress c-myc should provide a powerful tool for identifying myc's apoptotic targets.

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