

Review

Cell cycle molecules and vertebrate neuron death: E2F at the hub

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Abstract

Vertebrate neuron cell death is both a normal developmental process and the catastrophic outcome of nervous system trauma or degenerative disorders. Although the mechanisms of such death include an evolutionarily conserved core apoptotic pathway that is highly homologous to that first described by Horvitz and co-workers in *Caenorhabditis elegans*, it appears that many instances of neuron death additionally require the transcription-dependent induction of proapoptotic molecules. One such proapoptotic transcriptional pathway revealed by studies over the past decade revolves about the transcription factor E2F and those molecules that either regulate E2F activity or that are direct or indirect transcriptional targets of E2F. Many of the molecules associated with the E2F apoptotic pathway in postmitotic neurons also participate in the cell cycle in proliferating cells. Observations in human material and in animal and cell culture models show widespread correlation between changes in expression, activity and subcellular localization of E2F-related cell cycle molecules and developmental and catastrophic neuron death. A variety of experimental approaches support a causal role for such changes in the death process and are beginning to indicate how the neuronal E2F pathway activates the core apoptotic machinery. The discovery and elaboration of the neuronal apoptotic E2F pathway provides abundant targets as well as small molecule candidates for potential therapeutic intervention in nervous system trauma and degenerative disease.

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Abbreviations: ALS, amyotrophic lateral sclerosis; cdk, cyclin-dependent kinase; CDKI, cyclin-dependent kinase inhibitor; d/n,

dominant-negative; HDAC, histone deacetylase; NGF, nerve growth factor; PCNA, proliferating cell nuclear antigen; pRb, retinoblastoma protein; SOD, superoxide dismutase

Developmental and catastrophic vertebrate neuron death and involvement of the core apoptotic pathway

Death of postmitotic neurons can be either a normal or catastrophic event. During vertebrate development roughly half of all neurons generated die,^{1,2} and this process ultimately appears to play an important and beneficial role in the appropriate matching of pre- and postsynaptic elements. However, outside of development, loss of neurons – as for example due to physical injury, stroke, anticancer treatments or neurodegenerative disease – is a catastrophic event with clear and often debilitating consequences. Understanding the molecular mechanisms that underlie neuron death thus provides both insight about nervous system development and the opportunity for clinical intervention in a number of neurological disorders.

Among the remarkable and fundamental aspects of the groundbreaking work by Horvitz and coworkers on the molecular mechanism of apoptotic cell death is that the same molecular families that govern death in *C. elegans* also participate in death of vertebrate neurons. Thus, as elaborated in the accompanying review by Putcha and Johnson,³ homologs of ced3 (caspases), ced9 (Bcl2 family), ced4 (APAF1) and Egl-1 (BH3 domain family) represent the core machinery that regulates the distal portion of the neuronal apoptotic pathway.

In addition to the shared evolutionarily conserved core elements that govern apoptotic cell death in *C. elegans* and vertebrate neurons, there are also important distinctions. In the developing worm, specific individual cells are targeted to die based on genetically preprogrammed expression of the core apoptotic machinery. In contrast, developmental death of vertebrate neurons is a stochastic process involving selection within apparently equivalent populations of cells. This selection is largely based on access/lack of access to extrinsic signals that either promote or prevent neuron death. Examples include apoptosis elicited by the lack of access to neurotrophic factors that would otherwise prevent neuron death,^{1,2,4} and death evoked by proapoptotic ligands such as proneurotrophins.⁵ Thus, death occurs by extinguishing survival pathways (such as those involving Akt activation) and/or by permitting activation of pathways that promote death.

A role for transcriptional pathways in vertebrate neuron apoptosis

To promote death, the absence of trophic support or the presence of proapoptotic ligands must be linked to the evolutionarily conserved core death machinery by appropriate intracellular signaling pathways. Research over the last decade or so has provided the important observation that in a wide variety of cases, activation of the core apoptotic machinery in developing vertebrate neurons requires *de novo* transcription.^{6–10} This key discovery has in turn inspired the search for those transcription factors that mediate neuronal death and for the signaling pathways by which such factors are activated. To date several major transcription factors have been found that play required roles in propagating apoptotic signals in neurons. These include c-Jun,^{7,11} the p53 family,¹² Forkhead-FOXO¹³ and E2F. Once such transcription factors are activated in response to apoptotic stimuli, they regulate genes that ultimately interact with and engage the core apoptotic machinery, thereby driving neuron death.

Although the transcriptional component of neuron death was originally considered in the context of normal development, it has become increasingly clear that this mechanism also applies to the catastrophic death associated with neuronal injury and disease. Our aim in this review will be to focus on one particular proximal transcriptional pathway that regulates neuron cell death, namely that involving cell cycle molecules and E2F. Since E2F appears to be at the hub of this pathway, we will refer to it as the 'E2F pathway'. As will be reviewed here, ample correlative and direct experimental evidence ties the E2F pathway to neuron death in both developmental and catastrophic neuron death. In this review, we will discuss the intracellular events and associated molecules by which apoptotic stimuli engage E2F in the neuronal death process and the mechanisms as well as the consequences of this involvement. We shall also consider how this pathway is linked to activation of the core apoptotic machinery, and how interference with this pathway might be clinically relevant to preventing neuron death associated with injury, stroke and neurodegenerative disorders.

Cell cycle molecules and E2F as regulators of vertebrate neuron apoptosis

Initial findings implicating cell cycle molecules in neuron death

A variety of studies with non-neuronal cells have supported the idea that there is an overlap between the molecules that regulate the cell cycle and that promote apoptotic death.¹⁴ Observations that forcing quiescent cells into the cycle can induce their death seemed to be particularly relevant to that most quiescent of cells, the postmitotic neuron.¹⁵ By the early to mid-1990s, several authors raised the notion that cell cycle molecules might participate in neuron death.^{16–21} Material support came from the discoveries that transcripts encoding cyclin D1, a regulator of the cell cycle G₁/S transition, are induced in cultured sympathetic neurons targeted to die by withdrawal of NGF,¹⁸ and that induction of cyclin D1 protein precedes death of cerebellar granule cells in *lurcher* and

Table 1 Altered expression/activity of cell cycle molecules associated with neuron death

Identity	Response to apoptotic stimuli in neurons
<i>Cyclins</i>	
Cyclin D1	Elevated, nuclear translocation
Cyclin B	Elevated
Cyclin E	Elevated
<i>Cyclin-dependent kinases</i>	
Cdk4	Elevated, activated
Cdk1	Elevated, activated
Cdk2	Elevated, activated
<i>Cyclin-dependent kinase inhibitors</i>	
p27/Kip1	Decreased
p16/Ink4a	Decreased/elevated
<i>Pocket proteins</i>	
pRb	Hyperphosphorylated, degraded
p107	Hyperphosphorylated, degraded
p130	Hyperphosphorylated, lost from nuclei
<i>E2F proteins</i>	
E2F1	Elevated
E2F	Derepression of E2F-regulated genes Limited activation of E2F-responsive genes

staggerer mutant mice.²⁰ Further evidence arose from the finding that expression of SV40 T-antigen, which sequesters cell-cycle-related pocket protein family members, causes apoptotic death of postmitotic Purkinje cells.^{16,22} Additional reinforcement came from findings that proliferative inhibition by a ras dominant-negative (d/n) construct or by a variety of small molecule cell cycle inhibitors rescued cultured neuronal cells from apoptotic stimuli.^{17,23} Agents that block mitosis (when tested in proliferating cells) at or before the G₁/S transition were differentially effective, thus highlighting this particular part of the cycle.²³

In the interval since these 'early' beginnings, much evidence has accrued to implicate cell cycle molecules in neuron death. However, although a substantial and growing number of mammalian cell cycle molecules have been described, only a subset have been linked to neuron death at this time. These include primarily cyclins, cyclin-dependent kinases (cdks), cdk inhibitors, Rb family members, E2Fs and E2F target genes (see Table 1). In the discussion to follow, we shall consider each of these classes of molecules in turn and review the evidence for their involvement in both developmental and catastrophic death of neurons. We shall then present an integrated model for how such molecules regulate neuron survival and death. We shall also discuss how the neuronal apoptotic E2F pathway interfaces with other transcriptional death pathways and the core apoptotic machinery. Finally, we shall briefly consider potential clinical approaches to preventing catastrophic neuron death suggested by present knowledge of the apoptotic E2F pathway.

Cyclins and neuron death

Cyclins are essentially activating regulatory subunits for a series of kinases (cdks) that coordinate the cell cy-

cle.^{24,25} Each cyclin is generally present only during a particular portion of the cycle and has specific cdk partners. The five major cyclins, their cognate cdk and the cycle phases with which they are associated in viable proliferating cells are as follows: cyclin A (cdk2; S phase and cdk1; G₂/M), B (cdk1; G₂/M), D (cdk4 and cdk6; G₁), E (cdk2; G₁/S) and H (cdk7; all phases). The means by which levels of individual cyclins are regulated during different phases of the cycle in proliferating cells, and how such regulation in turn coordinates orderly progression of the cycle has been detailed elsewhere.^{24,25} What is particularly relevant about viable neurons is that they are nonmitotic and thus out of the cycle in the phase before G₁, designated as G₀.

Cyclin D1

One of the first indications that a cell is moving into the cycle from G₀ is the appearance of G₁-associated cyclin D. For this reason, cyclin D is a potentially major player in any cell-cycle-related event in neurons. There are three D-type cyclins, designated D1, D2 and D3. Of these, almost all present literature on neuron death concerns cyclin D1. As noted above, an early finding in the short history of this field was that NGF deprivation induces cyclin D1 transcripts.¹⁸ Since then, neuronal levels of cyclin D1 transcripts and/or protein have been reported to be elevated in response to a wide range of apoptotic stimuli *in vitro*, *in vivo* and in neurodegenerative diseases. For culture systems, this includes cerebellar granule neurons switched from culture medium containing depolarizing levels of K⁺ to one containing low levels of K⁺^{26–29} (we shall henceforth refer to this model as ‘repolarization’), cerebellar granule neurons exposed to kainic acid,³⁰ cortical neurons exposed to beta amyloid protein,³¹ sensory neurons exposed to cisplatin³² and serum-deprived neuroblastoma cells.³³ In the repolarization model, the distribution of cyclin D1 also appeared to change from a diffuse cytoplasmic pattern to a nuclear localization.²⁷

In vivo death models in which cyclin D1 protein and/or transcripts are elevated in affected neurons include spinal cord injury;³⁴ cisplatin treatment;³⁵ ischemia–reperfusion injury of the brain,^{36–38} retina,³⁹ or spinal cord;⁴⁰ lurcher and staggerer mutant mice;²⁰ kainic acid administration^{38,41,42} and motoneuron death in mice expressing a mutant form of superoxide dismutase (SOD) linked to amyotrophic lateral sclerosis (ALS).⁴³ Finally, cyclin D1 is reported to be aberrantly expressed in Alzheimer patients in those neurons that are susceptible to the disease⁴⁴ and in motoneurons of patients with sporadic ALS.⁴⁵

For a number of the neuron death paradigms listed above, cyclin D1 was induced at the transcriptional level. Surprisingly, the mechanisms by which apoptotic stimuli lead to induction of cyclin D1 in neurons are unknown and it will be important to address this issue in future to provide a full understanding of the apoptotic E2F pathway.

In addition to transcriptional induction, nontranscriptional mechanisms may also come into play with respect to elevation of neuronal cyclin D1. Boutellier *et al.*²⁹ reported that in the cerebellar granule neuron repolarization model, cyclin D1 protein accumulation is due to diminished proteasomal degradation caused by repolarization and inactivation of a

Ca⁺⁺/calmodulin-dependent pathway. At present, it is unclear whether such a mechanism also pertains to apoptotic paradigms that do not involve repolarization.

Although there is much correlative evidence to suggest that upregulation of cyclin D1 is causally involved in neuron death, only limited direct evidence has been generated thus far to test this hypothesis. Ino and Chiba⁴² reported that continuous infusion of cyclin D1 antisense (but not of sense) oligonucleotides into the lateral ventricles suppressed neuron death evoked by kainic acid. A cyclin D1 antisense construct also protected cultured cortical neurons from death promoted by beta amyloid protein.³¹ As reviewed below, a growing body of evidence links cdk4 to neuron death, thus implicating (though not directly demonstrating) involvement of cyclin D.

Cyclin E

In addition to cyclin D, cyclin E has been associated with neuron death in both *in vitro* and *in vivo* models and in disease. Cyclin E levels are elevated in beta-amyloid-treated cortical neurons³¹ and in cultured cerebellar granule neurons induced to die by kainic acid treatment⁴⁶ or by repolarization.²⁷ Elevated cyclin E has also been detected in neurons of Alzheimer disease patients.⁴⁷ Such findings would be consistent with movement of the affected neurons towards the G₁/S boundary. Moreover, cyclin E expression is regulated by a mechanism sensitive to the elevation of cyclin D⁴⁸ and could well be a consequence of the latter. Other than evidence for involvement of cdk2 (a cyclin E-associated cdk) in certain death paradigms (see below), data directly linking cyclin E to neuron death are presently lacking.

Cyclin B

Cyclin B has been reported to be elevated in cultured sympathetic neurons stimulated to die by exposure to dopamine^{49,50} and in cultured retinal ganglion cells induced to die by nerve growth factor-promoted activation of the p75 neurotrophin receptor.⁵¹ Cyclin B induction was not detected in either model. In addition, cyclin B is elevated in neurons affected by Alzheimer disease^{44,52–55} as well as in a number of neurodegenerative disorders including Down’s syndrome, progressive supranuclear palsy and Pick’s disease⁵² and in cerebrovascular disease.⁵⁶ Considering the association of cyclin B with the G₂/M phase of the cell cycle, such observations have led to the suggestion that in some cases neurons advance as far as G₂/M before death.^{44,51–55,57–59}

There is a growing number of reports that apoptotic stimuli can induce neurons, at least in some cases, to enter S phase as indicated by BrdU incorporation, mitotic figures and markers such as the proliferating cell nuclear antigen (PCNA).^{20,22,31,46,60,61} However, despite the attractive idea that inappropriately expressed cyclin B may play a role in neuron death, there are presently few experimental findings to support this. As reviewed below, the best support at present comes from the cerebellar granule repolarization model in which cdk1, a cyclin B-activated cdk, has been implicated.

The mechanism by which neuronal cyclin B is induced in response to apoptotic stimuli has not been studied. However, in cycling cells, like cyclin E, cyclin B expression

is downstream of events triggered by cyclin D1 and E2F (see below) and it thus appears possible that a similar mechanism may pertain to neurons.

Cdks

Cdks are serine–threonine kinases that are activated by cyclins at specific points in the normal cell cycle and that play a required role in regulating orderly movement from one stage to the next.^{24,25} The anticipated consequence of cyclin induction in neurons subjected to apoptotic stimuli is activation of their cdk partners and, where monitored, this indeed has been the case. Such activation appears to play an important role in a variety of paradigms of both developmental and catastrophic neuron cell death. We will consider here cdks 1,2,3,4 and 6. Although cdk5 is a member of the cdk superfamily and appears to play a role in neuron survival and death,⁶² there is little evidence that it directly contributes to the cell cycle and hence we will not consider it here.

Cdk4/6

These cyclin D-regulated cdks promote movement of cells from G₀ and G₁ toward G₁/S and would thus be among the first indicators that postmitotic neurons reactivate portions of the cell cycle machinery.²⁴ Cdk4 levels are relatively unchanged in proliferative cells in which the major means of regulation of activity occurs via regulated expression of cyclin D.²⁴ In contrast, levels of cdk4 protein appear to be relatively low in viable neurons. In PC12 cells, which are converted by nerve growth factor (NGF) from proliferating neuroblast-like cells to a postmitotic sympathetic-neuron-like phenotype,⁶³ cdk4 transcripts and protein are dramatically downregulated with neuronal differentiation⁶⁴ (J Angelastro and LA Greene, unpublished data). Withdrawal of NGF, which promotes apoptotic death in this system, results in a rapid re-expression of cdk4 (J Angelastro and LA Greene, unpublished data). Additional *in vitro* models of neuronal death in which cdk4 is upregulated include cisplatin-treated sensory neurons,³² beta amyloid-treated PC12 cells and cortical neurons,⁶⁵ and cerebellar granule neurons subjected to repolarization.²⁸ Furthermore, elevated cdk4 has been reported in neurons in animal models of spinal cord injury,³⁴ kainic acid-induced excitotoxic stress,⁴² transient spinal cord ischemia,⁴⁰ stroke³⁷ and ALS.⁴³ Neurons destined to die in Alzheimer disease also show cdk4 protein induction.^{44,66} Corresponding elevation of cdk6 has not been examined in most cases and was absent in the ALS model.⁴³ The latter finding raises the possibility that cdk6 contrasts with cdk4 in its response to apoptotic stimuli, although this point warrants further examination in additional models. For this reason, we will refer mainly to cdk4 here, but it should be kept in mind that cdk6 may also be involved in neuron death.

In nearly all cases in which both were examined, elevation of cdk4 accompanied elevation of cyclin D1 expression. This would be anticipated to lead to enhanced cdk4/cyclin D-associated kinase activity. This has been directly confirmed in several models including DNA damage of cultured cortical neurons,⁶⁷ repolarized cultured cerebellar granule neurons^{27,28,61,68} and in spinal cord of the mouse ALS model.⁴³

Additionally, as discussed below, Rb family members, which are major cellular substrates for activated cdk4, show enhanced phosphorylation in a number of neuronal death models.

The key question raised by such observations is whether activation of cdk4 can play a role in neuronal death. One approach to this issue has been the employment of small molecule cyclin-dependent kinase inhibitors (CDKIs). Of these, one of the most effective has been flavopiridol, a cell permeant inhibitor with selectivity for cdks 1,2 and 4.⁶⁹ At concentrations at which flavopiridol blocks cdk4 activity *in vitro*, it rescues cultured neurons from a wide variety of apoptotic stimuli including NGF withdrawal,⁷⁰ beta amyloid exposure,⁶⁵ cytosine arabinoside,^{67,71} camptothecin⁷² or UV treatment;⁶⁷ repolarization;²⁷ the electron transport chain inhibitor 3-nitropropionic acid⁴¹ and proteasome inhibitors.⁷³ Flavopiridol also proved to be protective in an animal stroke model.³⁷ Although supportive, the drawback of such studies is that, as with many small molecules, there is the issue of flavopiridol's specificity that appears to include at least several additional targets that might also affect neuron survival.⁷⁴ On the positive side, flavopiridol, which is currently in clinical trials as an anticancer agent,^{69,75} appears to be remarkably effective in promoting neuron survival, is well tolerated in patients and thus it or a related compound may prove to be clinically useful as a neuroprotective agent.

An alternative strategy to test the role of cdk4 in neuron death has been to interfere with its function or activity. One approach to this has been to overexpress the CDKI proteins p16/Ink4, p21/Waf and p27/Kip1 in neurons. CDKI proteins bind to and interfere with the kinase activity of cyclin–cdk complexes.^{76,77} Each of these proved to protect cultured neurons from apoptotic stimuli including NGF deprivation,⁷⁸ the DNA-damaging agents camptothecin, ara-C and UV⁶⁷ and exposure to proteasome inhibitors.⁷³ In addition, overexpression of p16/Ink4 protected murine neuroblastoma cells from death caused by serum deprivation³³. Significantly, p16/Ink4 is selective for activated cdk4/6 complexes, thus indicating the importance of these cdks in the apoptotic mechanism. Another approach has been to overexpress d/n forms of cdks in neurons. Sindbis-virus delivered d/n cdks 4 and 6 protected cultured neurons from NGF deprivation,⁷⁸ DNA damage,⁶⁷ beta amyloid⁶⁵ and proteasome inhibitors.⁷³ D/n forms of cdk3 were not protective in these models nor was d/n cdk2 except in the case of proteasome inhibition.⁷³ siRNAs and antisense constructs are additional potential means to interfere with cdk expression. Transfection of a cdk4 siRNA represses death of cultured neurons evoked by DNA damage (DX Liu, SC Biswas and LA Greene, unpublished data), while infusion of a cdk4 antisense oligonucleotide suppressed *in vivo* neuron death triggered by the excitotoxin kainic acid.⁴²

To summarize, both correlative and experimental evidence supports a role for cyclin D1 and cdk4 in death of diverse neuron types as triggered by various stimuli. However, in view of the potential lack of specificity of currently employed small molecule inhibitors and of possible unanticipated interactions of CKIs and d/n cdks with unintended targets, additional approaches such as the expanded use of siRNAs and antisense constructs would appear to be useful. It will also be important to further extend such studies to

animal models of developmental and catastrophic neuron cell death.

Cdk2

In proliferating cells, cyclin E–cdk2 complexes are activated at the G1/S interface.^{24,25,77} In addition to the elevation of cyclin E expression cited above, several pieces of evidence implicate cdk2 in at least some paradigms of neuron death. Cyclin E-associated kinase activity was elevated in repolarized cultured cerebellar granule neurons,²⁷ and cdk2 activity was induced in striatal neurons following *in vivo* mild cerebral ischemia.⁷⁹ With regard to functional evidence for involvement in death, d/n cdk2 constructs protected cultured neurons from death induced by beta amyloid³¹ (but see Giovanni *et al.*⁶⁵) and proteasome inhibitors.⁷³ In each of the models cited here, there was also evidence for functional involvement of cdk4, suggesting that both cdk4 and cdk2 can contribute to the death mechanism in the same neurons. However, as noted above, d/n cdk4 provided neuronal protection in several death models in which d/n cdk2 was ineffective, indicating that cdk2 activation is not obligatory for neuron death in all cases.

Cdk1

Cdk1 (also referred to as cdc2) appears during the G₂/M phases of the cycle in proliferating cells and is activated by cyclin B.^{24,25,77,80} Cdk1 is a transcriptional target of E2F1²⁵ and is repressed by E2F4/p130 complexes.⁸⁰ As elaborated below, this means that cdk1 may be a downstream target of the events triggered by elevation of cyclin D1 and activation of cdk4.

Like cyclin B, cdk1 is upregulated in affected neurons in Alzheimer disease⁵³ as well as in neurons affected by a number of additional neurodegenerative disorders.⁵² Immunostaining with a specific antibody for phosphoepitopes further indicates the presence of activated cdk1 in AD neurons.⁵³ Cdk1 is also elevated in experimental neuron death models including Purkinje cells of transgenic animals expressing T-antigen,⁸¹ dopaminergic substantia nigral neurons induced to die *in vivo* by axotomy or by treatment with 6-hydroxydopamine,⁸² and cerebellar granule cells undergoing *in vivo* developmental cell death or repolarization-induced death *In vitro*.⁸³

Aside from a correlative association, experimental evidence for involvement of cdk1 in at least one paradigm of neuron death arises from the observation that transfection of cultured cerebellar granule cells with d/n cdk1 or with a cdk1 antisense oligonucleotide suppressed apoptosis caused by repolarization.⁸³ In this model, a cdk2 antisense was reported to be ineffective in preventing death. Interestingly, examination of a different model of cerebellar neuron death (growth factor withdrawal) indicated no involvement of cdk1.⁸⁴ It thus will be important in future to test the extent to which cdk1 plays a functional role in additional neuron death paradigms.

The mode by which cdk1 elevation/activation can promote neuron death has also come under consideration. Immunohistochemistry has revealed the presence of shared phosphoepitopes in proliferative M-phase cells and in the neurofibrillary tangles that characterize, and that are thought

to contribute to the pathology of, Alzheimer disease.^{53,59,85} It has thus been suggested that activated cdk1 phosphorylates targets such as tau protein and nucleolin that in turn contribute to neurofibrillary tangles and to neuron degeneration in Alzheimer disease.^{53,59,85} The study of repolarized cultured cerebellar granule cells revealed a rather different mechanism by which cdk1 contributes to neuron death. In this case, the BH3 domain proapoptotic BAD was a target for cdk1. Phosphorylation of BAD by cdk1 led to its movement to mitochondria where it appeared to participate in the death mechanism by binding to BclXL,⁸³ thereby contributing to activation of the core apoptotic mechanism.

Small molecule cdk inhibitors – evidence but not proof for cdk involvement in neuron death

Olomoucine and roscovitine are purine analog cdk inhibitors that have neuroprotective actions in a variety of *in vitro* and *in vivo* neuron death models.^{27,51,70,79,86–88} Although such findings have been interpreted as supporting a role for cdk activity in neuron death, as in the case of flavopiridol, these molecules have less than absolute specificity⁸⁹ and hence such conclusions, while suggestive, must be viewed with caution and as subject to verification by alternative experimental approaches.

CDKIs: do they have a role in neuron survival and death?

As reviewed above, there are several classes of naturally occurring CDKIs that have been exploited to probe the functional roles of active cdk in neuron death. The protective actions of transfected, exogenous CDKIs suggest that sufficiently high levels of endogenous CDKIs could also serve to protect neurons from death even in the face of cdk activation in response to apoptotic stimuli; conversely loss of endogenous CDKIs might permit elevated cdk activity and neuron death. In this light, it is of interest that levels of the CDK1 p27/Kip1 fell substantially in cultured cerebellar granule neurons after repolarization^{27,28} and in cultured neocortical neurons subjected to oxygen–glucose deprivation.⁷⁹ In addition, p16/Ink4a is lost from striatal neurons induced to die *in vivo* by mild cerebral ischemia.⁷⁹ Of additional relevance, immature CNS neurons of mice null for the CDKIs p19/Ink4d and p27/Kip1 re-entered the cell cycle and underwent mitosis and this was accompanied by elevated levels of neuron death.⁹⁰ Such findings support the idea that CDKIs play an important role in suppressing cdk activity in postmitotic neurons, and that they function to prevent cell cycle re-entry as well as cdk-dependent apoptosis. The limited number of present observations strongly indicates that further experimental studies are in order to test whether loss of CDKIs is a general response to apoptotic stimuli and is required for neuron death.

An additional intriguing observation regarding CDKIs is that p16/Ink4 levels (as well as those of cdk4) are apparently elevated in neurons of Alzheimer disease patients.⁶⁶ A possible interpretation of this is that p16 elevation is a compensatory attempt to protect these neurons and that this

explains, at least in part, the relatively slow loss of neurons in the disease.

Rb/pocket proteins and neuron death

The apparent widespread involvement of activated cdk4, 6 and perhaps 2, 4 and perhaps 6 in neuron death raises the question of the identities of their relevant substrates. In proliferating cells, major targets for these G1/S kinases are members of the Rb (also designated pocket protein) family (see Stevaux and Dyson⁹¹ for review). The three members of this family are pRb, p107 and p130. Pocket proteins bind to members of the E2F family of transcription factors and act as repressors of E2F-mediated gene expression. There are at least six E2F proteins of which E2F1-5 interact with pocket proteins. There is a degree of specificity in these interactions in that pRb appears to bind E2F1-5, while p107 and p130 preferentially bind E2F4 and E2F5. Pocket proteins inhibit expression of E2F-regulated genes by two mechanisms. One is by binding to and directly blocking the E2F activation domain. The other is by recruiting chromatin-modifying proteins (such as histone deacetylases, methyltransferases and Polycomb group proteins) to E2F-binding sites, thereby causing direct gene repression. The way in which cdk4/6 come into this picture is that when they phosphorylate pocket proteins, the latter lose their capacity to bind E2F proteins. The consequences of this are that E2F is liberated to promote gene activation and that repressive E2F–pocket protein complexes are lost, resulting in gene derepression. The roles of activation and derepression of E2F-regulated genes to the cell cycle and to cell death will be discussed in a later section below.

Observations in a variety of systems indicate that cdk4/6 activation evoked by apoptotic stimuli does indeed lead to neuronal pocket protein phosphorylation. Stimuli including camptothecin,⁹² cisplatin,³² beta amyloid,³¹ repolarization^{27,68} and proteasome inhibitors⁷³ promote pRb phosphorylation in cultured neurons. Similar findings pertain to p107.⁹² In the cases tested, such Rb phosphorylation was blocked by the cdk4/6 inhibitor flavopiridol.^{73,92} Phosphorylation of Rb/p107 was followed by a substantial decline in total Rb/p107 protein and this effect was blocked by caspase inhibitors.^{68,92} Such observations indicate that activation of cdk4/6 induces Rb/p107 phosphorylation in neurons, and that this in turn permits their caspase-dependent degradation.

Elevated pRb phosphorylation has also been reported to occur in animal models of neuron death including stroke,³⁷ SOD-induced ALS⁴³ and kainic acid-induced excitotoxic stress.⁴¹ Similar observations have been made in affected neurons of patients afflicted with Alzheimer disease⁹³ and ALS.⁴⁵ In the ALS model, phospho-Rb coimmunoprecipitated with cdk4, but not the noncycle-related cdk5,⁴³ while pRb phosphorylation in the stroke model was blocked by flavopiridol.³⁷ Such findings support a role for cdk4 in the observed *in vivo* phosphorylation of pRb.

Although initial observations did not indicate effects of apoptotic stimuli on the third member of the pocket protein family, p130,⁹² re-examination in cultured neurons reveals that DNA-damaging agents and NGF deprivation do indeed affect this protein. Cellular subfractionation shows that hypophosphorylated p130 is present in nuclei, whereas the

hyperphosphorylated forms of p130 reside in the cytoplasm. In response to apoptotic stimuli, the hypophosphorylated nuclear p130 is rapidly lost while the cytoplasmic forms are little if any changed (DX Liu and LA Greene, unpublished data). This change was blocked by flavopiridol, consistent with a requirement for cdk4/6 activation.

One would anticipate that the functions of pocket proteins would be substantially altered by the effects of apoptotic stimuli on their phosphorylation, levels and subcellular localization. If this is the case, then do such changes play a role in neuron apoptosis? One strong indication of this is the observation that targeting SV40 T-antigen to Purkinje neurons *in vivo* causes their death.^{16,22,92} T-antigen binds pocket proteins and disrupts their interactions with endogenous partners such as E2F proteins. In support of such a mechanism, T-antigen that was mutated to inhibit its capacity to bind pocket proteins did not affect Purkinje cell survival.²² If functional loss of pocket proteins promotes neuron death, then their replacement should be protective. To test this, neurons have been transfected with excess pRb or with phosphorylation-resistant pRb mutants. Such constructs protect cultured neurons from diverse stimuli such as DNA-damaging agents, NGF deprivation and proteasome inhibitors.^{73,92,94}

The role of pocket proteins in neuron death and survival raises the question as to which member(s) of the family participate in this action. Most attention has been given to pRb; this protein has been widely studied for its role in the G₁/S transition^{25,77,91} and it is present in postmitotic neurons.⁹⁵ Although early reports with pRb-null mice suggested a role for pRb in neuron survival, recent studies indicate that loss of this gene causes defects in cell cycle, but does not promote death of postmitotic neurons.^{96,97} In line with the low levels of p107 in neurons,^{98,99} targeted deletion of this gene in mice yields no observable effect on neuron survival.¹⁰⁰ In addition, recent work indicates that pRb and p107 are not required for survival of postmitotic Purkinje neurons.¹⁰¹ By contrast, p130 is at relatively high levels in neurons^{98,102} and p130-null mice show massive strain-dependent neuronal death.¹⁰³ Thus, existing data appear to favor an important role for p130 in survival of postmitotic neurons.

We recently undertook a series of experiments to further identify the specific pocket protein(s) that regulate survival and death in neurons (DX Liu and LA Greene, unpublished data). Examination of E2F-binding sites in cultured neurons showed a large predominance of complexes with p130 and little presence of pRb or p107. These E2F–p130 complexes were lost in response to apoptotic stimuli as was nuclear p130. To test whether E2F–p130 complexes play a role in neuron survival and death, p130 expression in cultured neurons was downregulated by antisense and siRNA approaches; this resulted in apoptotic death. A functionally impaired p130 that displaces endogenous p130, but cannot promote gene repression also induced neuron death. In contrast, there was no effect of knocking down pRb expression. Finally, transfection of cultured neurons with a p130 mutant resistant to phosphorylation by cdk4 provided strong protection from NGF deprivation and DNA damage. Taken together, such observations support the hypothesis that p130 is the major pocket protein species that regulates neuron survival and death.

E2F at the hub of neuron death: the case for gene derepression

As discussed above, there are two major consequences of cdk-promoted phosphorylation and loss of cellular/nuclear pocket proteins. These are release of pocket proteins from the E2F transactivation domain, thereby permitting E2F-dependent gene induction, and dissolution of E2F–pocket protein repressor complexes, thereby promoting gene derepression. To distinguish between the activation and derepression mechanisms, neurons have been transfected with mutant E2F1 protein that retains the capacity to bind DNA, but lacks the activation domain. When overexpressed, this construct should displace endogenous E2F complexes from DNA, irrespective of their association with pocket proteins. Moreover, on one hand the mutant E2F should block E2F-mediated gene activation and on the other promote derepression of genes that are repressed by E2F–pocket protein complexes. Thus, the mutant should be protective if neuron death depends on E2F-mediated gene activation, but should compromise survival if death is due to E2F-mediated gene derepression. When tested in several different systems of cultured neurons, the mutant construct promoted death, indicating a derepression mechanism.^{94,104}

Additional experimental observations further support a mechanism of neuron death driven at least in part by derepression of E2F-regulated genes. A ‘decoy’ DNA construct consisting of multiple copies of the E2F-binding site also effectively promoted death of neurons.⁹⁴ This DNA would be expected to compete with endogenous DNA for binding of E2F and E2F–pocket protein complexes and to promote survival in the case of a transcriptional activation mechanism and death in the case of a derepression mechanism. Furthermore, derepression of E2F responsive reporters occurred in neuronal cells not only (as expected) with the E2F activation mutant and binding site decoy, but also in response to apoptotic stimuli.⁹⁴

If neuron survival requires E2F–pocket protein-mediated gene repression, how does this occur and what happens in response to apoptotic stimuli? Recent findings indicate that pocket proteins bind, in addition to E2F, chromatin-modifying proteins such as histone deacetylases (HDACs) and methyltransferases.⁹¹ When E2F–pocket protein complexes bind DNA, the tethered HDACs and methyltransferases modify nearby chromatin-associated proteins such as histones and by this means gene transcription is repressed. Examination of p130 in viable neurons revealed that it binds at least two such chromatin modifiers, HDAC1 and the methyltransferase Suv39H1; moreover, this association is lost in response to apoptotic stimuli (DX Liu and LA Greene, unpublished data). The importance of these pocket protein-bound modifiers in both gene repression and maintenance of neuron survival was indicated by findings that HDAC inhibitors such as trichostatin A cause both derepression of E2F-regulated genes and neuron death.¹⁰⁵ Similar results were achieved when neurons were transfected with a d/n form of methyltransferase Suv39H1 (DX Liu and LA Greene, unpublished data). Such observations support a mechanism in which E2F–pocket protein complexes repress proapoptotic genes in viable neurons by serving as anchors for chromatin

modifiers and in which apoptotic stimuli lead to loss of such complexes and consequent derepression of proapoptotic genes.

Does E2F-dependent gene activation have a role in neuron death?

There are multiple reports in which overexpression of exogenous E2F1 causes neuron death.^{84,104,106–109} Overexpression of E2F1 also accelerated loss of T-antigen-expressing Purkinje cells in transgenic mice.⁸¹ Although such findings can and have been viewed as supporting an E2F-dependent gene activation model of neuron death, there are alternative interpretations. For instance, in addition to binding unoccupied E2F1 activation sites (if any such are available), excess free E2F1 could compete with DNA-bound E2F for pocket proteins or could displace DNA-bound E2F–pocket protein complexes. Either of these actions would promote derepression of E2F-responsive genes and, according to our hypothesis, neuron death.

There are additional observations that appear to indicate a role for E2F1-promoted gene activation in neuron death. E2F1 is upregulated in neurons subjected to apoptotic stimuli *in vitro*^{46,68,104,105,107,109} and *in vivo*³⁷ and is elevated in degenerating neurons of patients with Alzheimer disease and with Down’s syndrome.^{93,110} In addition, neurons treated with an E2F1 antisense^{68,104} as well as neurons cultured from mice null for E2F1^{107,109,111–113} are at least partially protected from diverse apoptotic stimuli. Although such data can be taken to support a mechanism of E2F1-dependent gene activation, here again an alternative interpretation is possible. The E2F1 gene is repressed during G₀ and derepressed when cells move into G₁/S.^{25,91} In proliferating cells, such upregulation of E2F1 leads to activation of genes required for S phase.^{25,91} In neurons, one can imagine that apoptotic stimuli promote, via activation of cdk4 and phosphorylation of p130, derepression of E2F1 transcription and that this in turn leads to accumulation of E2F1 protein which then participates in death by the mechanisms discussed above for overexpression of exogenous E2F1. That is, E2F1 may be an initial target of apoptotic-stimuli-promoted gene derepression in neurons, which then contributes to death by enhancing further gene derepression or by stimulating gene activation.

Although the relative roles that activation and derepression of E2F-responsive genes play in various paradigms of neuron death have yet to be fully worked out, there is at least one system in which activation appears to be of importance. Recent observations established that repolarization of cerebellar granule cells results in transcriptional activation of cdk1, which in turn phosphorylates and functionally activates proapoptotic BAD.^{83,84} A cdk1 d/n was protective indicating that cdk1 contributes to death in this neuron death model.⁸³ Further work identified the cdk1 promoter, which contains an E2F-binding element, and showed that this promoter was sufficient to drive elevated expression of a reporter in repolarized granule neurons.⁸⁴ Deleting the region containing the E2F-binding element did not raise the basal expression of the reporter construct, whereas mutation of this site suppressed the response to repolarization. Such findings appear

to rule out a derepression mechanism for regulation of cdk1 in this model and to favor E2F-mediated gene activation. Given the reported elevation of cdk1 and its activator cyclin B in a number of paradigms as reviewed above, it will be important to determine whether direct activation of the cdk1 gene by E2F plays a wider role in neuron death beyond the repolarization model. Of relevancy, although E2F1-mediated cdk1 expression was required for repolarization-induced death of granule neurons, cdk1 was not elevated in the same neurons induced to die by withdrawal of a growth factor and, in this case, did not appear to play a role in the death mechanism.⁸⁴

Given such findings, what are the relative roles of E2F-dependent gene derepression and activation in neuron death? The most appealing interpretation at this time is that derepression of E2F-regulated genes plays a major role in the neuron death mechanism; in at least some cases, this leads to elevation of E2F1 levels that contribute to the death process by transcriptional activation of targets such as cdk1 and perhaps, by driving further gene derepression. The extent to which these alternative mechanisms contribute to neuron death may well vary depending on neuron type and apoptotic stimulus.

What are the targets of the neuronal E2F apoptotic pathway?

Irrespective of the relative roles of E2F-mediated gene activation and derepression in neuron death, a key issue is the identities of the proapoptotic proteins that are regulated by these mechanisms. Presently, the verified targets of E2F activation/derepression in neurons are few and this is an area that should merit intensive investigation in the future. As reviewed above, one transcriptional target to emerge is cdk1, which contributes to death by phosphorylating BAD and promoting its translocation from the cytoplasm to mitochondria. Another apparent target is c-Jun. A variety of studies have reported that apoptotic stimuli lead to elevation of neuronal c-Jun levels and that when c-Jun is activated by phosphorylation (by c-Jun N-terminal kinases), it in turn stimulates transcription of genes that contribute to death.⁷ Supporting c-Jun as a target of the E2F pathway, camptothecin, a DNA-damaging agent that evokes neuronal apoptosis, induced c-Jun in cultured neurons, and this response was blocked by cdk inhibitors such as flavopiridol and olomoucine and substantially suppressed by d/n forms of cdk2 and 6.¹¹⁴ In contrast, there was no effect of these reagents on c-Jun phosphorylation. These intriguing observations thus establish a potential link between two major transcriptional pathways that regulate neuron apoptosis. In this case, proapoptotic targets of activated c-Jun should also be indirectly dependent on activation of the apoptotic E2F pathway. On a cautionary note, given the possible nonspecific actions of small molecule cdk inhibitors and of d/n cdk2s, it will be desirable in future to confirm such findings by alternative approaches. In addition, it will be important to establish whether c-Jun induction is downstream of the E2F pathway in other paradigms of neuron death.

B- and c-myb are transcription factors with proapoptotic activity that are also regulated by E2F.¹¹⁵ Myb promoters

contain E2F-binding sites, and myb expression is subject to E2F-dependent repression.¹¹⁶ Application of apoptotic stimuli to cultured neurons results in upregulation of B- and c-myb transcripts as well as protein.^{11,94} A variety of approaches indicate that this is due to relief of E2F-mediated repression⁹⁴ that is mediated by binding of E2F-p130 to the myb promoter (DX Liu and LA Greene, unpublished). The possibility that B- and/or c-myb regulated in this manner may play a role in neuron death is supported by the observation that overexpression of exogenous B- or c-myb in neurons results in apoptosis.⁹⁴ Mybs are transcription factors and so the question arises as to what these regulate in turn that promote neuron death. One promising myb target is BIM, a proapoptotic member of the BH3 family that is upregulated in neurons in response to NGF deprivation^{117,118} and downregulated by NGF when it induces proliferating PC12 cells to become nonmitotic sympathetic-neuron-like cells.¹¹⁹ The BIM promoter contains several myb-binding sites and a reporter construct driven by this promoter (but not one with mutated myb sites) shows elevated expression in response to apoptotic stimuli in neurons (SC Biswas, DX Liu and LA Greene, unpublished data). Moreover, pharmacologic and molecular interference with the E2F pathway totally blocks elevation of neuronal BIM expression in response to apoptotic stimuli (SC Biswas, DX Liu and LA Greene, unpublished data). Since BIM induction in neurons is partially dependent on the c-Jun pathway,¹¹⁷ it may be that blockade of the E2F pathway inhibits BIM expression by a combination of effects on expression of c-Jun and of mybs.

As recounted here, there are many variations on the theme of how the E2F pathway promotes neuron death. In this light, although there has been progress in identifying targets of E2F-dependent gene activation and derepression that play causal roles in neuron death, it seems highly likely that many more remain to be identified. Advances in generating neuron death models and in detecting changes in gene expression should greatly facilitate this important objective.

Putting it all together: a unified model of how the E2F pathway regulates neuron survival and death

We have reviewed here evidence that proteins otherwise associated with the cell cycle also participate in mediating neuron cell death under a variety of circumstances and that E2F is at the hub of this mechanism. Putting it all together, the following sequential scheme emerges (see Figure 1). In the absence of apoptotic stimuli, viable neurons are in G₀ and their levels of cyclin D as well as of cdk4 are low. E2F proteins are complexed by pocket proteins and this results in silencing of E2F-responsive genes, both by inhibition of gene activation and by active gene repression. The latter requires chromatin modifiers that are tethered to the complex. The identities of all the components in the repressor complexes are not known, but include p130, HDAC1 and Suv39H1. The E2F proteins that are involved are unknown at present, but a likely contributor is E2F4, which is a preferred partner for p130 and which is highly expressed in neurons.^{1,91,120} Such complexes inhibit expression of genes needed for cell cycle entry as well as genes that promote cell death. In response to

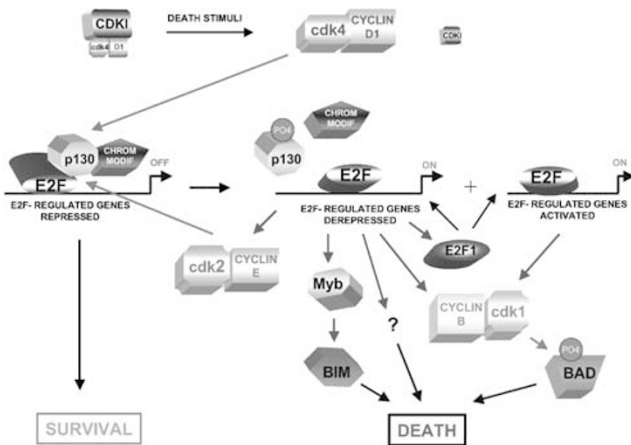


Figure 1 Schematic representation of the apoptotic neuronal E2F pathway

apoptotic stimuli, levels of cyclin D1 and cdk4 become elevated and as a consequence, neuronal cdk4 activity is greatly enhanced. Activated cdk4 (and perhaps also cdk6) then phosphorylates pocket proteins (of which p130 seems to be the most relevant), which in turn causes dissociation of E2F–pocket protein–chromatin modifier complexes, degradation/cytoplasmic translocation of pocket proteins and loss of gene repression. In what presently appears to be a cell and apoptotic-stimulus-dependent manner, derepressed genes include cyclins E and B, E2F1 and B- and c-myc. Elevated cyclin E activates cdk2 that contributes further to pocket protein phosphorylation and dissolution of E2F–pocket protein complexes. E2F1 activates transcription of cdk1 which, when activated by cyclin B, phosphorylates BAD, thereby permitting the latter to translocate to mitochondria. B- and c-myc induce transcription of BIM. Both phospho-BAD and BIM participate in release of mitochondrial proteins such as cytochrome *c*, which activate postmitochondrial elements of the core apoptotic pathway. Cyclin B–cdk1 complexes may also phosphorylate cytoskeletal proteins and additional targets that contribute to neuronal degeneration and death. Elevation of c-Jun (which, when activated by phosphorylation, participates in neuron death) also appears to lie downstream of the E2F pathway, but the mechanism of this regulation is unclear. It seems likely that additional proapoptotic downstream targets of the pathway remain to be identified.

A few closing thoughts

On the complexity of neuron death pathways

There are several principles that have emerged from studies on how neurons die. One is that although there are several overarching mechanisms such as involvement of E2F, there is considerable variation in the details of how these play out depending on neuron type and apoptotic stimulus. A good example is the elevation of cdk1 that is associated with repolarization of cerebellar granule neurons, but not with withdrawal of a trophic factor from the same cells. It remains to be seen just how specifics of apoptotic stimulus and neuron type intersect with and drive such variations in the E2F pathway. A second principle is that neuronal apoptotic stimuli

often activate diverse death pathways at the same time. Thus, several different transcriptional pathways can be triggered in neurons by apoptotic stimuli, including those dependent on c-Jun, E2F, p53 and FOXO. Moreover, as reviewed here for E2F, even a single such pathway can launch multiple proapoptotic events. One possible reason for such functional redundancy is to insure that death is efficiently achieved. Alternatively, such complexity may be part of a ‘fail-safe’ system that requires at least several independent apoptotic pathways to be launched simultaneously to achieve neuron death. A third and related generalization is that neuron death appears to be a threshold event that can be reached either by very strong activation of a single transcriptional pathway or by the cumulative responses to multiple transcriptional pathways. Experimentally, neuron death can be driven by overexpression of suitable components of any one of the above pathways. Examples for E2F include overexpression of mybs or of the E2F-binding site decoy. However, the observation that blocking any one of the above transcriptional pathways is often sufficient to suppress death promoted by apoptotic stimuli suggests that under physiologic death conditions, no single pathway is sufficiently activated to reach the threshold for death. This may account for widespread expression of elevated E2F pathway molecules observed in neurodegenerative disorders. If such molecules were harbingers of rapidly advancing death, then one might expect to find very few living neurons with cell-cycle-related markers. If on the other hand, additional events, such as activation of p53, must occur to reach an apoptotic threshold, then neurons might persist for relatively long periods of time even though the E2F pathway is activated. A fourth and emerging concept is that multiple transcriptional neuronal apoptotic pathways may converge on single targets. A good case in point is BIM induction. This has been shown to be partially dependent on the JNK pathway¹¹⁷ and recent findings also show regulation by FOXO.¹²¹ As mentioned here, BIM additionally appears to be a target of the E2F pathway (SC Biswas, DX Liu and LA Greene, unpublished observations). Such convergence may reflect a mechanism by which multiple transcriptional pathways contribute to reach a threshold level of expression of proapoptotic molecules.

The E2F pathway as a target for therapeutic intervention in catastrophic neuron death

The widespread ectopic expression of cell cycle molecules in susceptible neurons in patients with neurodegenerative disorders and in a variety of disease-related models, coupled with experimental evidence for causative roles of such molecules in neuronal apoptosis, has led many to propose the E2F pathway as a target for therapeutic intervention in catastrophic neuron death.^{41,52,58,122–127} A major challenge is to find means to block the pathway without interfering with normal cell proliferation. One approach is to continue to identify additional pathway components. There is no doubt that such components exist and there are already several intriguing candidates that, due to limited study and length considerations, have been omitted from this review. While the complexity of the E2F pathway and its branch points poses a

challenge for those wishing to understand this mechanism in depth, it also provides opportunities by multiplying the number of potential therapeutic targets and thereby, the chances for developing agents that selectively promote neuron survival.

A second approach to therapeutics is to exploit existing agents. It is ironic that most effort toward targeting cell cycle molecules has taken place in the context of neoplasias. It may well be that antiproliferative agents designed to kill rapidly cycling cells will also block neuron death. One example is flavopiridol that is currently in clinical trials for cancer.^{74,75,128} Although the exact mechanism by which this cdk inhibitor protects neurons is unclear, it does appear to represent a powerful starting point to design drugs that block the E2F pathway and that may repress catastrophic neuron death. Many additional agents have been developed that affect the E2F pathway and it would seem to be of high priority to assess these and derivatives for neuroprotective activity.

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