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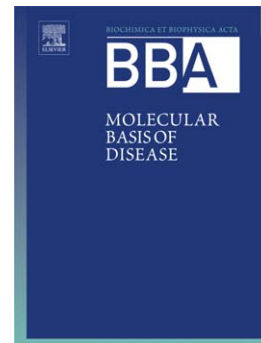
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Cell Cycle Molecules Define A Pathway Required For Neuron Death In Development And Disease

Lloyd A. Greene¹, David X. Liu, Carol M. Troy and Subhas C. Biswas

Department of Pathology and Center for Neurobiology and Behavior, Columbia University College of Physicians and Surgeons, 630 W. 168th Street, New York, NY10032

¹Correspondence at Department of Pathology, Columbia University Physicians and Surgeons, 630 W. 168th Street, New York, NY 10032. Tel: 212-305-6369. Email: Lag3@columbia.edu. Fax: 212-305-5498.

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Abstract

We review here evidence defining a molecular pathway that includes cell cycle-related molecules and that appears to play a required role in neuron death during normal development as well as in disease and trauma. The pathway starts with inappropriate activation of cyclin dependent kinase 4 (Cdk4) in neurons which leads to hyper-phosphorylation of the pRb family member p130. This in turn results in dissociation of p130 and its associated chromatin modifiers Suv39H1 and HDAC1 from the transcription factor E2F4. Dissociation of this complex results in de-repression of genes with E2F binding sites including those encoding the transcription factors B- and C-Myb. Once elevated in neurons, B- and C-Myb proteins bind to the promoter for the pro-apoptotic BH3-only protein Bim and promote its induction. Bim then interacts with the core cellular apoptotic machinery, leading to caspase activation and apoptotic death. This pathway is supported by a variety of observations and experimental findings that implicate it as a required element for neuron loss in development and in many nervous system traumas and disorders. The components of this pathway appear to represent potential therapeutic targets for prevention of disease-associated neuron death.

Introduction and overview.

As recounted by reviews in this special issue and elsewhere [1-12], cell cycle-related molecules appear to play key roles in neuron death associated with development and disease. Our aim here will be to describe the components of a molecular pathway in which cell cycle elements participate in neuron death. We will relate how activation of a cyclin-dependent kinase in neurons triggers a chain of molecular events that ultimately engages the core apoptotic machinery and culminates in cellular demise.

There are six overarching themes of this article. The first is that there are striking and mutually informative similarities between the molecular mechanisms that govern neuron death during normal development and in neurological disorders. The second is that neuron death in both instances involves similar changes in the activities and expression of molecules otherwise associated with the cell cycle. The third is that the pathway includes the sequential activation of cyclin-dependent kinase 4 (Cdk4); hyper-phosphorylation of the Rb family member p130; dissociation of p130 and associated chromatin modifiers from the transcription factor E2F4, resulting in gene de-repression; de-repression of the genes encoding the transcription factors B- and C-Myb; enhanced occupancy by B- and C-Myb of the promoter for the pro-apoptotic BH3-only protein Bim; and activation by Bim of the core apoptotic machinery. The fourth theme is that neuron death mediated by this pathway is not simply due to an aborted attempt to traverse the cell cycle, but rather reflects alternative actions of molecules that are also involved in cell proliferation. Fifth, although this pathway is necessary for neuron death under many conditions, it is not sufficient and works in conjunction with additional signaling pathways to mediate apoptosis. Lastly, our understanding of the specific molecules that contribute to neuron death in disease provides targets for novel therapeutic opportunities.

Cell cycle molecules and developmental neuron death.

During normal vertebrate development, approximately 50% of all post-mitotic neurons that are born undergo cell death [13]. An abundance of evidence supports a model in which the decision of which neurons live or die is regulated by their competition for limiting amounts of target-derived trophic factors [14-17]. The best studied of these trophic factors with respect to neuronal survival and death is nerve growth factor (NGF). In *in vitro studies*, largely (but by no means exclusively) carried out with rodent sympathetic neurons and PC12 pheochromocytoma cells have revealed a wealth of information about what happens in nerve cells when they are deprived of NGF and how this results in their apoptotic death. This has been strongly complemented by *in vivo* and *in vitro* studies with other trophic factor systems and models.

Among early observations relevant to our theme was that NGF deprivation in sympathetic neuron cultures was followed by elevation of transcripts encoding cyclin D1 (CycD1), a regulator of cyclin dependent kinases 4 and 6 (Cdk4/6) [18]. In proliferation-competent cells, an increase in CycD1 protein levels triggers movement from the G0 or G1 phase of the cycle into G1/S [19,20]. It was also reported that the apoptotic stimulus of serum deprivation in cultures of neuronally differentiated murine neuroblastoma cells resulted in activation of CycD1-dependent cdk activity [21]. Moreover, blockade of this activity by over-expression of the cdk inhibitory protein p16INK4A1, was protective. Additional evidence linking loss of trophic support to dysregulation of G1/S markers and other cell cycle proteins came from a genetic model in which post-mitotic cerebellar granule cells die due to loss of their synaptic targets and presumptive source of trophic support [22]. In this model, neuron death was also associated with elevation of cyclin D and other cell cycle-related proteins.

If G1/S-related events are required for developmental neuron death, it follows that agents that interfere with the G1/S transition should be protective. Indeed, blockade of this transition with a dominant-negative form of Ras or with a variety of small chemical G1/S inhibitors effectively rescued neuronal cells from NGF deprivation [23,24].

The potential involvement of cyclin D1 in developmental neuron death implicates its partners cdks 4 and 6. These play important roles in permitting replication-competent cells to undergo the G1/S transition [19,20]. One test of the potential importance of these proteins in developmental cell death has been the employment of small chemical cdk inhibitors such as olomoucine, roscovitine and flavopiridol. These have proved to be remarkably effective in blocking neuronal apoptosis evoked by trophic factor deprivation [25-29]. Although widely employed, one weakness of such drugs is that they lack specificity both within the cdk family and with respect to other kinases [30-33] and this has complicated the mechanistic interpretation of their actions in neuron death. An alternative approach has been the delivery of constructs encoding naturally occurring cdk inhibitor proteins such as p21, p16(INK4A1), and p27(kip1). Over-expression of these proteins protects neurons from trophic factor deprivation, reinforcing a role for cdks in neuronal developmental cell death [21,34]. To test the roles of specific cdks in neuron death, dominant-negative forms of these proteins have been expressed in neurons. It was observed that virally-delivered dominant-negative forms of Cdk4 and 6, but not of Cdk2 and Cdk3 protected sympathetic neurons from NGF deprivation [34]. Because of the potential capacity of dominant negative constructs to interact with and inhibit shared targets and regulators, we have more recently used shRNAs to specifically knock down Cdk4 [35]. This too has protected against NGF deprivation, further implicating this partner of CycD1 as a required element in the mechanism by which loss of trophic support triggers apoptotic death.

Although Cdk4 has multiple potential targets, its major substrates during cell proliferation are members of the Rb family of “pocket” proteins, namely pRb, p107 and p130 [19,20]. (In this review, we will use Rb to refer to the entire family and pRb to indicate this specific family member). In proliferating cells, hyper-phosphorylation of Rb by Cdk4 is an important event in the G1/S transition. In accordance with the involvement of Cdk4 activation in developmental neuron death, all three Rb family members rapidly undergo hyper-phosphorylation in response to trophic factor deprivation [28,36].

Although initial studies focused on pRb and its role in survival and death of post-mitotic neurons [37,38], more recent findings suggest that this protein may be more involved in regulating proliferation of neuronal precursor cells than in maintaining neuron survival [39,40]. Our findings suggest that the family member that is most important for regulating neuron survival and death is p130 [36]. In support of this, electrophoretic mobility shift assays (EMSA) indicate that relevant E2F-binding DNA complexes formed in post-mitotic neurons contain mainly p130 rather than pRb or p107. Moreover, the importance of p130 in neuron survival is underscored by the observation that knockdown of p130, but not of pRb or p107 induces neuron death [36].

How does p130 maintain neuron survival and how does its hyper-phosphorylation by Cdk4 result in developmental neuron death? An important (but not the only) activity of Rb family proteins is to associate with members of the E2F transcription factor family [19,20]. Such complexes have two roles. One is to suppress E2F's transactivating activity and the other is to repress genes to which they are bound. Hyper-phosphorylation of Rb by Cdk4 causes such complexes to dissociate. This has two potential consequences: release of E2F so that it can now transactivate suitable targets and de-repression and induction of genes otherwise silenced by E2F-Rb complexes [19,20].

In response to trophic factor deprivation, as described above, p130 undergoes hyper-phosphorylation. It appears that a major effect of this is translocation of p130 out of the nucleus [36]. This presumably further reduces its capacity to participate in gene repression. EMSA, chromatin immunoprecipitation (ChIP) and luciferase reporter assays all confirm that the associations of p130 with its DNA targets are lost in response to NGF deprivation and that this requires cdk activity [36].

As noted above, loss of p130-E2F complexes due to hyper-phosphorylation by activated Cdk4 permits both elevated E2F transactivational activity and de-repression of genes with E2F binding sites. Of these, multiple lines of evidence point to the latter as the most relevant to developmental neuronal death [41]. For instance, a decoy DNA construct with multiple E2F binding sites that effectively soaks up endogenous E2F is sufficient to trigger neuron death. This is consistent with the de-repression mechanism of death in that sequestration of E2F should result in loss of E2F-p130 complexes from DNA. On the other hand, if death were due to E2F-driven transactivation, such a construct would be (but is not) protective.

Another supportive finding is that constructs encoding phosphorylation-resistant Rb mutants or fusion proteins between E2F and Rb, which should selectively bind to and silence genes with E2F binding sites, effectively protect neurons from NGF deprivation [36,41]. As discussed below, additional experiments show de-repression of specific death-associated genes when NGF is withdrawn.

There is partial insight as to how p130 represses genes that lead to developmental neuron death. In addition to associating with E2F, Rb family members simultaneously bind molecules that remodel chromatin [36]. Thus, within complexes with E2F, Rb family members serve as tethers for proteins that modify chromatin and that promote gene silencing. Two such p130-associated proteins have been identified in neurons, histone deacetylase (HDAC1) and Suv39H1 [36]. Both are associated with p130 in healthy neurons and both are lost from complexes with p130 and E2F on DNA under conditions of NGF withdrawal [36]. Suv39H1 (suppressor of variegation 3-9 homolog 1) is a histone methyltransferase that transfers methyl groups to lysine 9 on histone H3 [42] while HDAC1 deacetylates histones [43]. Both modifications promote gene repression. As evidence of its role in maintaining neuron survival, loss of cellular Suv39H1 or transfection with an Suv39H1 mutant that binds p130 but lacks methyltransferase activity causes both gene de-repression and neuron death [36]. In addition, inhibition of HDAC activity also leads to gene de-repression and to neuron death [36,44,45]. Finally, although E2F-p130 fusion constructs protect neurons from NGF deprivation, such constructs that are defective in binding HDAC and Suv39H1 lack protective activity [36]. There are other chromatin modifiers in addition to HDAC1 and Suv39H1 that could conceivably play roles in p130-dependent neuron survival [cf. 46] and it will be of interest to identify these in future studies.

Like the Rb family, the E2F family consists of multiple members and there appears to be a selectivity for the association between specific Rb and E2F proteins [19,20,47,48]. In particular, the preferred partners for p130 are E2F4 and E2F5. Our findings indicate the most relevant partner for p130 in maintaining neuron survival is E2F4 [36]. E2F4, but not other E2F family members were found to occupy the promoter of the E2F-repressed pro-apoptotic gene *B-Myb* in healthy neurons (see below for further discussion) and was bound there to p130, but not pRb or p107. In addition, knock-down of E2F4, as in the case of p130, is sufficient to trigger apoptotic neuronal death. Taken together, such findings support the view that intact E2F4-p130 complexes and their associated chromatin modifiers are required to maintain neuron survival and that in trophic factor deprivation, such complexes are lost, resulting in de-repression of genes that promote death.

A key issue regards the identities of the death-associated genes that are repressed by p130-E2F4 complexes to maintain neuron survival. One important family of such molecules has been identified to date, the B- and C- members of the Myb family of transcription factors. *B-* and *C-Myb* are classic examples of genes that possess binding sites for E2F and that are repressed by E2F-Rb family member complexes [49,50]. In response to cdk-dependent hyper-phosphorylation of Rb family members

in proliferating cells, B- and C-Myb are induced and play major roles in promoting cell cycle progression [49,50]. B- and C-Myb are also induced in neurons after NGF deprivation [41], but in this case, they promote apoptotic death. In healthy neurons, the promoters for *B-* and *C-myb* are occupied by p130-E2F4 along with Suv39H1 and HDAC1, and B- and C-Myb expression is suppressed. In response to NGF deprivation, E2F4-p130-Suv39H1/HDAC1 complexes are lost from at least the B-myb promoter and expression of Myb transcripts and protein is elevated [36]. This induction promotes death in that over-expression of B- or C-myb triggers neuronal apoptosis while suppression of B- or C-myb induction by siRNA or antisense constructs protects neurons from NGF deprivation [41,51]. These findings thus establish that B- and C-Myb are among the genes that are de-repressed in neurons when p130 is hyper-phosphorylated after NGF deprivation and that their expression is required for apoptotic death under such conditions.

Because Mybs are transcription factors, their role in neuron death most likely results from regulation of other genes. To date, we have identified one important and relevant target of Mybs in cells deprived of trophic support and this is the apoptosis facilitator molecule Bim/Bcl2-like 11. Bim is a pro-apoptotic member of the Bcl2 family that belongs to the subgroup of BH3-only proteins [52,53]. Current evidence indicates that Bim binds to and neutralizes the function of pro-survival proteins such as Bclx and Bcl2 and by this means promotes caspase activation and apoptotic death. Bim is induced in response to NGF deprivation and its knockdown partially protects neurons from withdrawal of trophic support [35,54-56]. Therefore, it appears to be among the regulated genes that are involved in promoting developmental neuron death. Two binding sites for Mybs have been found in the Bim promoter. Significantly, we found that mutation of these sites abolishes the induction of a Bim reporter construct that occurs in response to NGF deprivation [35]. Moreover, occupancy of these sites by at least C-myb is elevated by NGF withdrawal. Additional evidence that Bim is a target of Mybs comes from findings that induction of Bim by NGF deprivation is blocked by Myb shRNAs [35]. In line with the pathway described above, Bim induction via Mybs during NGF deprivation is dependent on Cdk4 and E2F-mediated gene de-repression [35]. Thus, Bim induction after NGF withdrawal is blocked by cdk inhibitors and Cdk4 shRNA and is suppressed by the E2F-Rb fusion construct described above that silences genes with E2F binding sites.

Considered together, present findings indicate that cell-cycle-related events that are triggered by loss of trophic support in neurons constitute a pathway that culminates in induction of at least one protein, Bim, with clear pro-apoptotic activity and in apoptotic death (see the scheme in Figure 1). To summarize, loss of trophic support in models of developmental neuron death leads sequentially to 1) activation of Cdk4; 2) hyper-phosphorylation of p130; 3) dissociation of complexes containing E2F4, p130, Suv39H1 and HDAC1; 4) de-repression of genes occupied by the latter complexes, including those encoding B- and C-myb; 5) accumulation of elevated levels of cellular B- and C-myb proteins; 6) binding of B- and C-myb to the promoter for the gene encoding the pro-apoptotic protein Bim; 7) induction of Bim protein; 8)

engagement and activation of the core apoptotic machinery by Bim via its neutralization of pro-survival Bcl2 proteins; 9) caspase activation and death.

Cell cycle molecules in neuron death associated with diverse apoptotic stimuli.

Although many of the mechanistic studies described above have focused on neuron death associated with NGF deprivation, it is important to emphasize that at least several aspects of this scheme appear to pertain to a number of additional apoptotic paradigms. For instance, many of the cell cycle pathway elements that characterize neuron death evoked by NGF deprivation also mediate death of both peripheral and CNS neurons caused by DNA damaging agents [51,57,58]. Components of this pathway have also been implicated death of various neuron types brought about by various insults including hypoxia [59,60], excitotoxic injury [61-63], physical trauma [64], proteasomal inhibitors [65-67], and withdrawal of support by hyperpolarizing levels of K⁺ [28]. Such considerations by no means imply that the pathway described here or variations that employ alternative sets of cycle-related proteins are universally involved in neuron death. Nevertheless, present data encourage us consider the apoptotic cell-cycle pathway in paradigms well beyond just the case of NGF deprivation. In this context, we next review evidence for the role of the apoptotic cell cycle pathway in neurological disorders.

Cell cycle molecules in neuron death associated with neurological disorders: parallels with developmental neuron death.

Several types of evidence have linked cell cycle molecules to neuron death in disease. The first are correlative observations that cell cycle-related proteins are elevated in neurons that are at risk to die in various neurodegenerative disorders, traumatic injuries and ischemia/stroke, either in human disease or models thereof. These findings have been extensively reviewed in this volume and elsewhere and so need not be recounted here in depth. However, of particular relevance is that these changes include the same molecules involved in developmental neuron death. For instance neuronal cyclin D is elevated in models of ischemia/stroke [58,59,68-73], ALS [74], Alzheimer disease (AD) [75], excitotoxic stress [67-69], and spinal cord injury [64] as well as in affected neurons in human patients with AD [76-79], ischemia [80] and sporadic ALS [81]. Likewise, neuronal Cdk4 expression is reported to be up in models of ischemia/stroke [58,73], excitotoxicity [62], ALS [74] as well as in neurons from patients with AD [76,82] and stroke [80]. Consistent with elevated cyclin D and Cdk4, phospho-pRb immunostaining in relevant neurons is increased in models of ischemia/stroke [59,83,84] and AD [85,86] and in patients with Parkinson's Disease (PD) [87], AD [79,88] and sporadic ALS [81]. With respect to Bim induction, this has been found for experimental optic nerve transection [89], seizure [90] and neonatal hypoxia/ischemia [91] and in human seizure [90]. Western immunoblotting of AD and control frontal cortex also revealed elevation of Bim protein in the former [92].

Although there has been little work on Mybs in neurodegenerative diseases, there is evidence for their de-repression in at least one human disorder, autoimmune paraneoplastic cerebellar degeneration (PCD). In this malady, the target antigen of auto-antibodies is pcd-17, a protein that blocks B-myb de-repression [93]. The pcd-17 auto-antibodies appear to enter Purkinje cells and block pcd-17 function and by this means, permit B-Myb de-repression and consequent death.

The second type of evidence that cell-cycle related molecules play roles in nervous system disease is based on experimental manipulation of their activity or expression in cell culture and animal models. Among the most widely used approaches is the application of small chemical Cdk inhibitors (generally olomoucine, roscovitine or flavopiridol, though others have also been employed) to disease models and the demonstration that these have ameliorative actions on behavior and/or neuron survival. Examples in which this approach has been successful include models of AD [64], ischemia/stroke [58,94], excitotoxic stress [63,95,96], Niemann-Pick Disease [97], traumatic brain injury [98], optic nerve transection [99], and spinal cord injury [100].

While the experimental use of such chemical inhibitors has supported the involvement of a cdk-dependent pathway in disease-related neuron death and may well point the way to clinically useful drugs, there are several serious reservations about this interpretation. One issue regards the specificity of such agents for cdk. As in the case of most drugs, one cannot rule out actions on other classes of enzymes, especially kinases, and indeed such activities have been described for flavopiridol, olomoucine and roscovitine [cf 30-33]. However, the fact that all three inhibitors seem to have similar actions in protecting neurons does argue that non-cdk actions are less likely.

A related concern is the selectivity of these agents for various cdk family members. Each of these drugs targets different cdk with variable levels of specificity. For instance, flavopiridol has been described as a “pan-cdk” inhibitor [32] and there are no published reports in which the actions of flavopiridol or other such inhibitors have been systematically evaluated on all known cdk. To compound the issue, accumulating evidence has implicated Cdk5, which is a target of many cdk inhibitors, in neuron death associated with neurological disorders [101-105]. This raises several possibilities. One is that cdk other than, or in addition to, Cdk4 are involved in disease-related neuron death. In this light, Rashidian et al. [84] have suggested that both Cdk4 and Cdk5 play complementary roles in neuron death in hypoxia/ischemia with Cdk4 regulating apoptotic-like death and Cdk5 promoting excitotoxic death. Another possibility is that cdk in addition to Cdk4 drive hyper-phosphorylation of Rb family members. It has been recently reported that activated Cdk5 can directly hyper-phosphorylate Rb in a neuronal cell death model [106]. If this finding holds for additional death paradigms, it may be that disease-related Cdk5-dependent neuron death is, at least in part, mediated via the phospho-p130/Myb/Bim pathway described here.

Owing to the potential limitations of present small chemical cdk inhibitors, alternative strategies are required to test the involvement of specific cdks in disease-associated neuron death. One approach has been the use of dominant-negative cdks. Studies with such reagents showed that dominant-negative forms of Cdk4 and 6 protected cultured neurons from death evoked by β -amyloid [86]. Because dominant-negative proteins may have non-selective actions such as interactions with substrates common for other cdks, we have employed a knockdown strategy with shRNAs. We find that an shRNA targeted to Cdk4 protects cultured neurons from β -amyloid [107], thus reinforcing the conclusion that at least in this model, Cdk4 is required for neuron death. This observation also supports the idea that Cdk4 mediates neuron death in neurodegenerative disorders such as AD in which it appears to be elevated and/or activated.

Our finding that knockdown of Cdk4 suppresses death evoked by β -amyloid has led us to further pursue the hypothesis that the same pathway identified in developmental cell death is also activated in AD. We found that neuron death in the cellular β -amyloid model was associated with induction of Bim message and protein and that shRNA targeted to Bim was highly protective [107]. Moreover, Bim induction as well as death were blocked in this system by knocking down B-Myb. Thus, in this cellular AD model, many of the same cycle-related mechanisms that characterize the death induced by NGF deprivation are present, including the requirement for Cdk4, hyper-phosphorylation of Rb proteins and the essential induction of B-Myb and Bim.

Indications that the above pathway mediates neuron death by β -amyloid in culture have led us to explore whether this might also occur in AD itself. As reviewed above, susceptible neurons in AD show elevated expression of cyclin D, Cdk4 and hyper-phosphorylated Rb. We find that a large proportion of such neurons also co-express elevated levels of Bim [107]. These findings thus establish that affected neurons in AD brains express both the distal and proximal elements of the death pathway we have defined in developmental neuron death and in cellular models of AD. We would anticipate comparable findings in additional neurological disorders.

Other cell cycle elements in neurological disease.

The molecules that constitute the pathway described here (cyclin D, Cdk4, phospho-Rb and Mybs) are normally associated with the G1/S transition. However, a potential complication is the somewhat bewildering observation that neurons at risk in a variety of disorders show simultaneous elevation of proteins that are considered to be markers of additional phases of the cycle in proliferating cells. How do these additional cell cycle proteins fit into the scheme presented here? A variety of approaches have revealed that E2F-Rb complexes, including E2F4-p130, bind to and regulate numerous cell-cycle associated genes [48,108]. In proliferating cells, hyper-phosphorylation of Rb and loss of such complexes permits induction of genes

that promote DNA synthesis and an orderly progression to the next stages of the cycle. In neurons, by contrast, hyper-phosphorylation and loss of E2F-Rb complexes will also lead to induction of multiple cell-cycle associated proteins, but in this case, leads to cell death. Although our suspicion is that many of the cell-cycle-related proteins that are induced as a result of neuron disease or injury are bystanders of p130 hyper-phosphorylation and play little if any role in death, others may contribute. For instance, up-regulation of cyclins B and E has been noted in AD [71,109,110]. These in turn activate Cdk1 and Cdk2, respectively both of which can target Rb family members [111,112]. Activation of these kinases may thus mimic or augment the death-promoting actions of Cdk4 in neurons. In addition such kinase may have other relevant targets. For instance, Cdk1 can target and activate the pro-apoptotic BH3-domain molecule BAD [2]. Another potential player is Cdk7 which phosphorylates and activates other cdks including Cdks 1,2 and 4. Cdk7 levels are reported to be elevated in susceptible neurons in AD [113].

The presence of markers for various stages of the cycle in disease-affected neurons has led to suggestions that such cells undergo an “aborted” or “dysregulated” cell cycle and that this is what is responsible for their demise, perhaps by triggering death-promoting check point controls. In our view, the critical event is not the attempt to synthesize DNA or to traverse the cycle, but rather the de-repression of genes such as B- and C-Myb that can have multiple functions in addition to promoting proliferation. For example, depending on the cellular context, B-Myb can induce either proliferation or cell survival [49,50] or, as reviewed here, neuron death [41,51]. As we argue below, the choice between these actions and the reason that Myb is fatal in a neuronal context, may depend on which additional transcriptional regulators are present.

Another point to consider regarding the relationship between cell cycle and neuron death in disease is that in our model, the key E2F and Rb family proteins involved are E2F4 and p130. Our culture experiments indicate that these are the major family members complexed with DNA and the Myb promoter in healthy post-mitotic neurons and that such associations are lost upon activation of Cdk4 and result in death. Present findings indicate that E2F4 and p130 are not generally involved in regulating cell proliferation (in contrast to E2F1-3 and pRb and p107) and that they appear at high levels in the nervous system when neurons become post-mitotic [114,115]. In neurological diseases, the consequence of losing E2F4-p130 complexes due to activation of Cdk4 may therefore be to trigger death rather than replication [see 47].

Neurological disorders and trophic factor deprivation.

We have drawn parallels here between the apoptotic pathways involved in neuron death associated with development and with disease. Observations that treatments such as exposure to β -amyloid can rapidly activate this mechanism suggest that it can occur independently of loss of trophic signaling. On the other hand, one

consequence of a number of neurodegenerative disorders and of nervous system trauma is impairment or loss of access to and/or retrograde transport of trophic factors [116,117]. Thus, the mechanism described here by which cell cycle molecules contribute to neuron death in neurological disorders may be driven both by direct apoptotic stimuli as well as by loss of trophic support.

The potential role of aggregated proteins and proteasomal dysfunction in activation of the apoptotic cell cycle pathway in neurodegenerative disorders.

Dysfunction of the ubiquitin-proteasomal system is an additional potential, and not necessarily mutually exclusive contributor to activation of the neuronal apoptotic cell cycle pathway. There is accumulation of aggregated proteins in a number of degenerative disorders and this may cause or be the result of reduced neuronal ubiquitin-proteasomal activity [reviewed in 118,119]. It has been further suggested that failure ubiquitin-proteasomal triggers neuron death and degeneration in such diseases [118,119]. The ubiquitin-proteasomal system plays a major role in regulation of cell cycle by degrading key proteins such as cyclins that promote transition from one phase of the cycle to another [reviewed in 120]. A relevant example is CycD which is degraded by the proteasomal system to prevent activation of cdks such as cdk4 and movement of cells into G1/S. Thus, one could imagine that a neuron with compromised proteasomal function might accumulate CycD as well as other potential activators of the apoptotic cell cycle pathway. Evidence to support this concept has come from experiments with cultured cerebellar neurons induced to die by removal of supporting levels of K⁺ or by treatment with proteasomal inhibitors [65]. Several additional studies have shown that neuron death induced by proteasomal inhibitors induce and require activation of the cell cycle machinery, including cdk4 [66,67]. Thus, irrespective of the initial cause, the reduction of ubiquitin-proteasomal function that occurs in neurodegenerative disorders could contribute to activation of the apoptotic pathway described here.

Cell cycle molecules are necessary, but not sufficient triggers of neuron death.

We have argued that activation of cell cycle molecules is necessary for neuron death in development and disease. However, they are unlikely to be sufficient in this regard. Even in systems in which there is strong evidence for an essential role of cell cycle molecules in neuron death, additional required pathways have been uncovered. A good example is the pathway dependent on activation of the c-Jun N-terminal protein kinases (JNKs). The JNK pathway has been implicated as required for neuron death associated with diverse causes including AD [121,122], PD [123], hypoxia/ischemia [124] and trophic factor deprivation [125,126]. At least part of the pro-apoptotic activity of JNKs appears to be due to their capacity to phosphorylate and activate the transcription factor c-Jun [126]. One aspect that the JNK pathway shares in common with the apoptotic cell cycle pathway described here is that it too can lead to activation of the pro-apoptotic protein Bim in neurons. This has been

observed thus far for NGF deprivation [55,127], ischemia [128] and β -amyloid exposure [S.C. Biswas and L.A. Greene, unpublished]. Moreover, induction of Bim by apoptotic stimuli in neurons is suppressed by inhibition of either the cell cycle or JNK pathways [35]. Thus, it appears that both pathways must be activated simultaneously to promote maximum Bim induction and death. Yet a third transcriptional pathway can also regulate Bim expression in neurons, and that is one involving the FOXO subfamily of Forkhead transcription factors [129]. FOXO appears to be involved in both Bim induction and in death evoked by NGF deprivation [129]. In the presence of NGF, FOXO proteins are phosphorylated by a PI3K/AKT-dependent pathway and are excluded from the nucleus; with NGF deprivation, de-phosphorylation permits FOXO movement to the nucleus and regulation of death-promoting genes including Bim [130].

If at least three independent transcriptional pathways appear to regulate Bim expression in neurons and if each plays a required role in death, how is this coordinated? Examination of the Bim promoter reveals conserved binding sites for Myb [35] and FOXO [129] proteins. In addition, we find that the promoter also possesses a binding site for c-Jun [S.C. Biswas and L.A. Greene, unpublished]. Thus, the Bim promoter contains regulatory binding sites for products of all three pathways (i.e. the cell cycle, JNK and FOXO pathway). We have further observed that inhibition of any one of the three pathways is sufficient to block NGF-deprivation-induced activation of a reporter driven by the Bim promoter [S.C. Biswas and L.A. Greene, unpublished]. These findings point to and support a model in which neuron death requires the convergence of multiple independent pathways to induce the pro-apoptotic protein Bim. That is, under physiologic conditions, each of the pathways appears to be required for Bim induction and for neuron death, but none is sufficient. For full Bim induction and for neuron death, all three pathways need to be activated simultaneously. One possible reason for the evolution of such a mechanism is that it acts as a “failsafe” to protect neurons from dying if only a single pathway is activated inappropriately. This consideration may also explain why Myb induction might lead to death in a neuronal context, but not in proliferating cells. That is, death would only occur if the complementary transcription factors were activated and this would not be the case for proliferating cells in which FOXO proteins are inactivated by growth-factor-dependent PI3K/AKT signaling. This convergence model is also consistent with the progressive loss of neurons that characterizes many neurodegenerative disorders. If neuron death in such diseases requires a threshold level of activation of pro-apoptotic proteins such as Bim, this would not occur until all three pathways were sufficiently activated.

Therapeutic perspectives

The characterization of a pathway that is dependent on defined cell cycle molecules such as Cdk4 and that is required for neuron death in disease opens the door to specific therapeutic intervention. One obvious approach that appears promising thus far in models of spinal cord injury [100] and ischemia/stroke [94] is the use of

small molecule cdk inhibitors. Issues such as specificity, side effects on proliferating tissues, and access to the nervous system must be considered before moving forward. Fortunately, mainly due to the potential use of such agents for treatment of cancers, efforts are underway to develop a variety of drugs that are selective for specific cdks [33,131]. However, a major, though presumably soluble, roadblock in evaluating such drugs for their neuroprotective actions in suitable cell and animal models has been their limited accessibility to the overall research community. Another issue is whether such agents might be useful for inhibition of neuron death in progressive neurodegenerative disorders. It remains to be seen whether even the most specific cdk inhibitors could be tolerated for prolonged periods of time and whether blockade of neuron death in these disorders is sufficient to yield therapeutic effects.

The model we have described here includes molecules in addition to cdks. This raises the possibility that these too might be considered as potential therapeutic targets. One advantage of blocking the death pathway at such levels is that they are upstream of activation of the core apoptotic machinery and thus of cellular degeneration due to mitochondrial dysfunction. Bim is attractive in this regard in that, based on the viability of Bim null mice, it appears to be dispensable for survival of the organism. Moreover, basal Bim levels in normal brain appear to be very low. Various strategies such as targeted siRNAs or antisense oligonucleotides or site-specific drugs that interact with Bim and block its interaction with anti-apoptotic Bcl2 family members could thus prove useful in sustaining neuron survival in disease.

It is not unlikely that the cell-cycle-related molecular pathway recounted here is more complex than presently understood and that it possesses additional components and regulatory features. Although this would further challenge our capacity to understand the mechanisms that govern neuron death in development and disease, identifying additional pathway components may also provide new opportunities and targets for therapeutic intervention.

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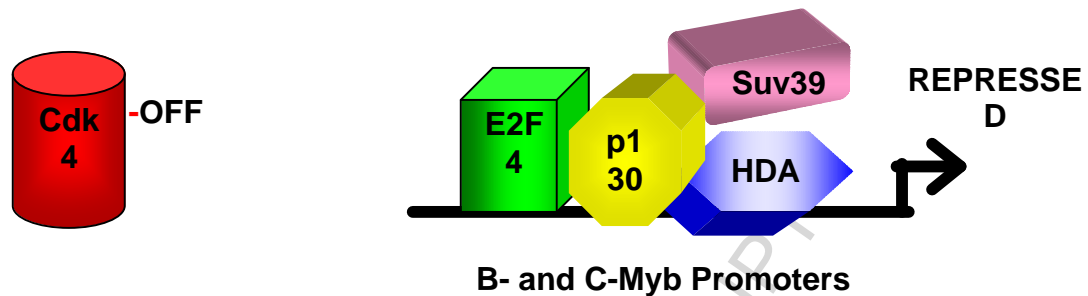
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Figure Legend

Figure 1: Scheme depicting a molecular pathway by which cell-cycle-related molecules promote cell death in neurons in development and disease. A. *Survival conditions*. Under conditions of neuron survival, p130 forms complexes with E2F4 on the promoters of genes including those encoding B- and C-Myb. p130 tethers chromatin-modifying proteins such as Suv39H1 and HDAC1 to the promoter and represses their transcription. Cdk4 is inactive and consequently p130 is hypo-phosphorylated and binds tightly to E2F4. B. *Apoptotic conditions*. Under apoptotic conditions, Cdk4 associates with Cyclin D and is active. This leads to hyper-phosphorylation of p130 which in turn causes it and its bound chromatin modifiers to dissociate from E2F4 and from promoters of genes including B- and C-Myb. This results in gene de-repression and elevation of cellular levels of B- and C-Myb. The Mybs bind to the promoter of the gene encoding the pro-apoptotic BH3-only protein Bim. The levels of intracellular Bim rise and it engages the core cell death machinery, promoting caspase activation and apoptosis.

A. SURVIVAL
CONDITIONSB. APOPTOTIC
CONDITIONS