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Interplay Between the p53 Tumor Suppressor Protein Family and Cdk5

Novel Therapeutic Approaches for the Treatment of Neurodegenerative Diseases Using Selective Cdk Inhibitors

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Abstract

Cyclin-dependent kinases (Cdks) play a key role in orchestrating the coordination of cell cycle progression in proliferating cells. The escape from the proper control of the cell cycle by the upregulation of cyclins or aberrant activation of Cdks leads to malignant transformation. In quiescent cells and/or terminally differentiated cells, the expression pattern and activity of Cdks is altered. In postmitotic neurons, expression of mitotic kinases is downregulated, whereas Cdk5 expression becomes upregulated. Similarly to other Cdks, free Cdk5 displays no enzymatic activity and requires complex formation with a specific regulatory subunit. Two activators of Cdk5 have been identified. p35 and its isoform p39 bind to, and thereby activate, Cdk5. Unlike mitotic kinases, Cdk5 does not require activating phosphorylation within the T-loop. Because p35 is a short-lived protein, the p35/Cdk5 complexes are unstable. The stability of the p35 protein is regulated by its Cdk5-mediated phosphorylation of p35. Activated p35/Cdk5 kinase phosphorylates numerous physiological targets.

The proper phosphorylation of the most important substrates, such as τ protein and neurofilament H, is essential for the correct regulation of the cytoskeletal organization, thereby regulating cell adhesion, motility, and synaptic plasticity. Moreover, Cdk5 regulates the activity of the p53 tumor suppressor via phosphorylation. p53 is upregulated in multiple neuronal death paradigms, including hypoxia, ischemia, and excitotoxicity, and plays a key role in the induction of apoptosis.

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On the other hand, an abnormally high expression and elevated activity of Cdk5 was observed in neurodegenerative diseases, suggesting the application of Cdk inhibitors for their therapy. Considering the action of some Cdk inhibitors on the expression and activity of the p53 protein, their therapeutic efficacy must be carefully evaluated.

Index Entries: Cdk inhibitors; cell cycle arrest; apoptosis.

Expression and Activity of Cyclin-Dependent Kinases in Proliferating Cells

Regulation of the cell cycle is based on a precise coordination of the activity of protein complexes consisting of cyclins and cyclin-dependent kinases (Cdks). Cyclins, which constitute the regulatory components of the complexes, are expressed in a cell-cycle-dependent manner. The activities of Cdks, the catalytic components, are regulated by several mechanisms, including their proper folding, binding to the corresponding cyclin, and site-specific phosphorylation of Cdks catalyzed by Cdk-activating kinases, and in the case of Cdk1, by its site-specific dephosphorylation by cell-cycle-controlled phosphatases. Moreover, the activity of cyclin/Cdk complexes can be negatively regulated by cellular Cdk inhibitors belonging to one of two protein families *(1)*. Numerous cyclins and Cdks have been identified in eukaryotic cells, several of which are essential for the control of specific transition points of the cell cycle (Fig. 1). The G_1/S and G_2/M transitions are key steps of the cell cycle. The most important event regulating the G_1/S transition is stepwise phosphorylation of the tumor suppressor pRb mediated by D-type cyclins/Cdk4/6 and cyclinE/Cdk2 as well as subsequent release of the E2F transcription factors, which facilitates the onset of the transcription of S-phase genes. In early S-phase, cyclin A/Cdk2 complexes promote the cell cycle progression, whereas cyclins A and B in complexes with Cdk1 control the G_2/M transition. Generally, the regulation of Cdk activity is based on the balance between activating phosphorylation, the association of Cdks with the corresponding cyclins, and the presence of cellular Cdk inhibitors.

Considering their crucial role in cell division, cell cycle regulatory proteins were believed to be indispensable for survival. Surprisingly, the inactivation of the D-type cyclins, cyclin A, cyclin B, Cdk4, and Cdk2 by gene disruption resulted in developmental defects in animal models, but they were not lethal.

Components of the Cell Cycle Machinery in Neuronal Cells

During terminal differentiation, neural cells exit the active cell cycle, enter G_o phase, and become quiescent. The differentiation of multipotent neural progenitor cells into neurons and glial cells appears to be accompanied by a restriction in the range of fates available to individual cells. The cell cycle exit represents the key event promoting differentiation of cells and acquisition of neural cell identity. Consequently, a novel program of gene expression must be induced. Although several cell cycle regulatory proteins are downregulated during neuronal differentiation, an elevation in the expression of cyclin D1 and Cdk5 has been observed. The downregulation of the cellular factors regulating the cell cycle in proliferating cells appears to be necessary, because re-expression and activation of distinct components of the cell cycle machinery such as Cdk2, Cdk4, or E2F have been detected in the brain of patients with Alzheimer's disease as well as in different conditions of neurodegeneration in animals *(2–4)*.

In some experimental models, cyclin D1 has been found to be increased during neural differentiation stimulated by inducers such as

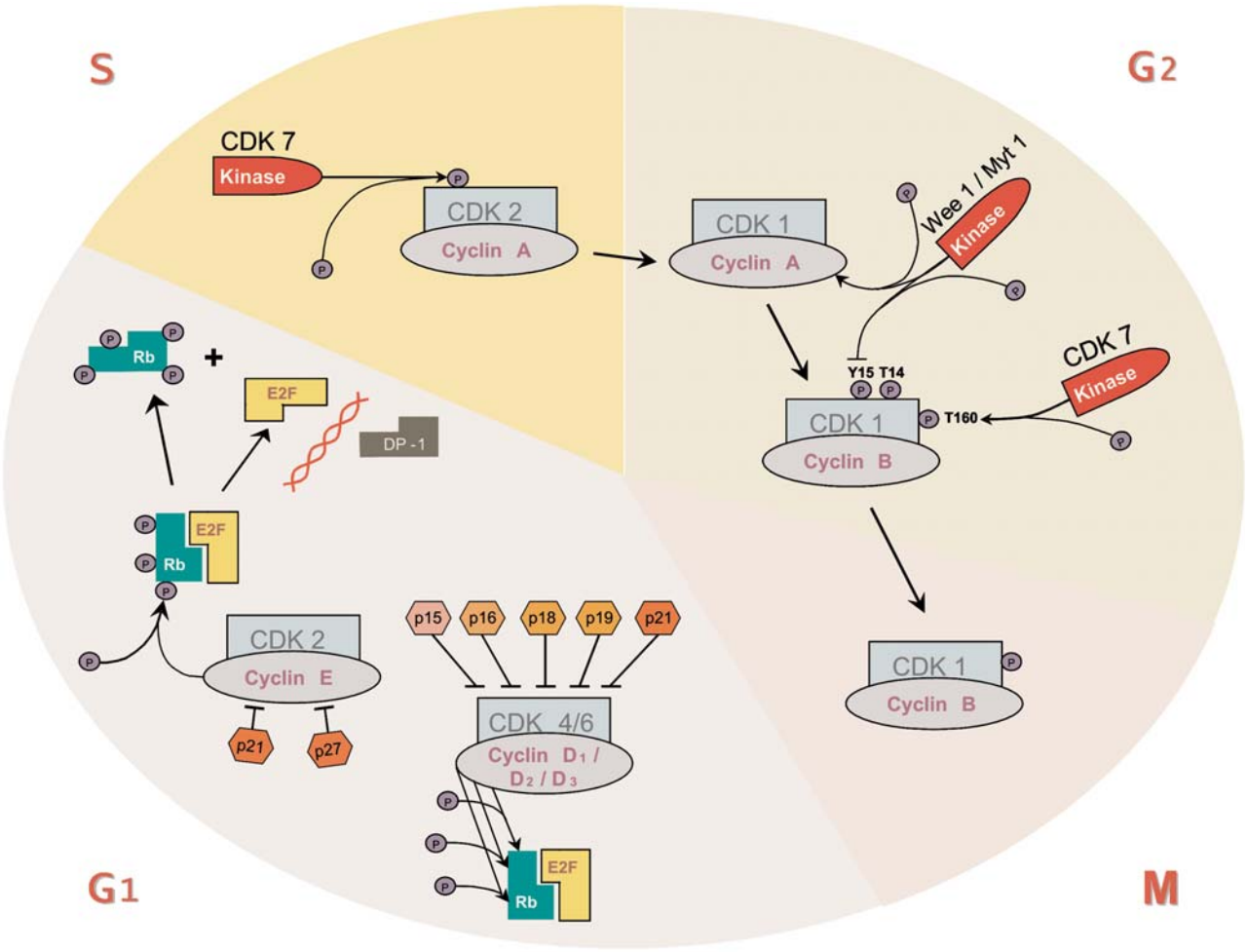

Fig. 1. Scheme of the regulation of cell cycle progression.

nerve growth factor (NGF) and fibrillary growth factor *(5–8)*. Despite an observed increase of cyclin D1 levels, the activity of cyclin D1/Cdk complexes was reduced *(8)*. Inactivation of cyclin D1 by gene disruption in mice resulted in severe neurological defects (e.g., abnormal limb reflex), resembling those that occur after disruption of genes involved in neuronal differentiation *(9)*. However, high expression of cyclin D1 *per se* was not sufficient to promote the neuronal differentiation *(10)*, indicating that elevation of cyclin D1 is not the trigger but rather an accompanying phenomenon of neuronal differentiation.

What is the role of cyclin D1 upregulation during neuronal differentiation? The exact function of cyclin D1 has not been elucidated. Cdk5 bound to cyclin D1 has no enzymatic activity. Cyclin D1 may sequester Cdk5 and maintain it in an inactive state until Cdk5 is able to form complexes with its specific modulators, p35/p39.

Cdk5, a highly homologous protein to the other Cdks, is ubiquitously expressed in different mammalian tissues. In postmitotic neurons, expression of Cdk5 becomes upregulated *(11,12)*. The importance of Cdk5 in neuronal development became evident after its inactiva-

tion by gene disruption *(13)*. Inactivation of the *Cdk5* gene was lethal, and Cdk5 knockout embryos died because of extensive abnormalities in the central nervous system (CNS), including cortical and hippocampal lamination defects and cerebrellar hypoplasia, thereby implicating involvement of Cdk5 in neuronal migration during development of the CNS.

Despite ubiquitous expression of Cdk5 in mammalian cells, its active form is primarily detectable in postmitotic neurons *(14)* because of the selective occurrence of the specific cyclin-like proteins p39, p35, and p25 (a truncated form of p35) in the nervous system *(15)*. Abnormal expression and aberrant activity of Cdk5 have been observed in some neurodegenerative disorders such as Alzheimer's disease and amyotrophic lateral sclerosis *(11,15)*.

Cdk5, which shows high homology to mitotic kinases—especially to Cdk1—is a proline-directed kinase that phosphorylates serine and threonine residues positioned directly upstream of a proline moiety. Therefore, the Cdk5 activity could be regulated in a manner similar to other kinases. Indeed, Cdk5 requires an association with a cyclin-like specific regulator. However, the activating phosphorylation by Cdk-activating kinase appears dispensable *(16,17)*.The phosphorylation of Thr160 (or Thr161) in the T-loop of Cdks is usually required for full activation. Ser159 in the T-loop of Cdk5 is in very close proximity to p25, and its phosphorylation would probably negatively affect the binding to p25 and, therefore, the kinase activity.

It appears that p35, a regulatory subunit of Cdk5, is critical for kinase activation *(18,19)*. Although p35 serves as a regulatory partner for Cdk5, it does not display any primary sequence homology to members of the cyclin family of proteins. However, the predicted tertiary structure of p35 is similar to that of cyclins *(20,21)*. p35 protein expressed exclusively in postmitotic neurons of the CNS forms a complex with, and therefore activates, Cdk5 *(18,22,23)*.

In studies, the overexpression of cyclin p35 in different cell lines induced neuronal differentiation. However, the co-expression of dominant-negative Cdk5 inhibited the terminal differentiation. This indicates that cyclin p35 promotes neuronal differentiation via activation of Cdk5. These results are consistent with the observations obtained in experiments performed on $p35^{-/-}$ mice. Animals lacking p35 exhibited defects in cortical lamination and fasciculation of axon fibers *(24–26)*, presumably because of aberrant neuronal migration *(24)*. p35, localized in the cell periphery (most likely in the plasma membrane) *(14,27)*, is a short-lived protein with a $t_{1/2}$ of 20 to 30 min in vivo (Fig. 2). It consists of an amino-terminal p10 region and a carboxy-terminal p25 domain that encompasses the Cdk5 binding site. The ubiquitin proteasome pathway has been demonstrated to mediate the fast turnover of p35 (Fig. 2; ref. *28*). Deletion of the amino-terminal part generates the stable p25 protein, indicating that the amino-terminal p10 region is necessary for degradation (Fig. 3). Further studies performed in distinct cell lines extensively investigated the regulation of the p35 steady-state. The stability of ectopically expressed p35 was reduced after cotransfection with wild-type *Cdk5* but not with the catalytically inactive kinase *(28)*. Moreover, the inhibition of the endogenous p35/Cdk5 kinase in neurons by roscovitine (ROSC) increased the stability of p35 *(28)*. These results strongly indicate that the kinase activation promotes p35 degradation. Indeed, p35 becomes autophosphorylated by p35/Cdk complexes *(28)*.

The final evidence for the negative feedback regulation of the p35/Cdk5 complexes was provided by deletion mutagenesis of the p35 protein. Single and combined mutations of four minimal consensus Cdk phosphorylation sites within the p35 protein (Fig. 2) revealed that the stability of these p35 mutants increased two- to threefold *(28)*. The greatest increase in stability was exhibited by the p35 combined mutant, in which all four potential phosphorylation sites were substituted with alanine *(28)*. These data substantiate the hypothesis that site-specific phosphorylation stimulates p35 degradation. Together, the

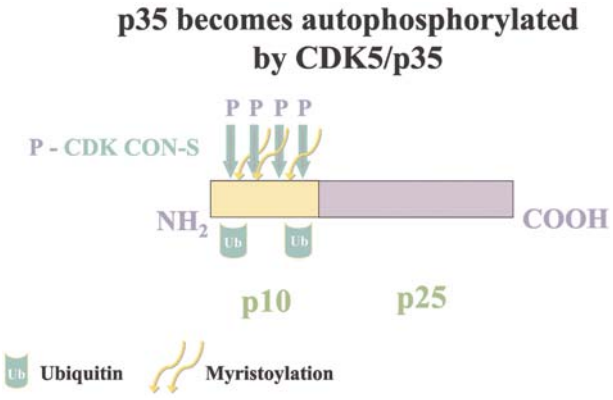

Fig. 2. Posttranslational modifications of the p35 protein regulating its stability. P-Cdk CON-S, consensus Cdk phosphorylation sites.

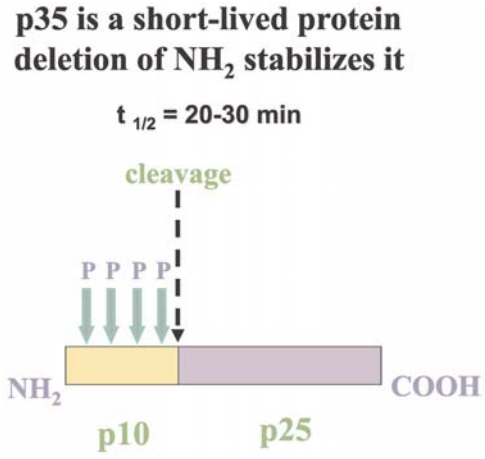

Fig. 3. Diagram depicting the structure of the cyclin-like protein p35, a specific regulator of Cdk5 activity and its cleavage site.

results mentioned earlier indicate that the activity of p35/Cdk5 complexes is unstable in normal neuronal cells and is very tightly regulated. Upon activation of Cdk5 by binding to p35, the kinase phosphorylates its own activator soon after it modifies its main substrates. Autophosphorylation induces, or at least facilitates, p35 degradation by the proteasome in a polyubiquitin-dependent manner.

The kinase activity of Cdk5 is additionally regulated by p39, another neuronal-specific cyclin. The primary structure of cyclin p39, which is localized primarily in cerebrum and cerebellum, exhibits about 50% identity with p35 *(29)*.

Physiological Function of p35/Cdk5 Complexes

The substrate specificity of the p35/Cdk5 complexes is similar to that of the mitotic kinases Cdk2 and Cdk1 that modify the K(S/T)PX(K/R) consensus sequence motif *(30,31)*, where S or T are serine or threonine residues, respectively, that are prone to phosphorylation. Numerous neuronal proteins have been proved as substrates for the enzymatic activity of p35/Cdk, including structural proteins such as neurofilaments, microtubule-associated protein τ, and mitogen-activated protein-2 *(32)*. Additionally, proteins associated with neural transmitter release (e.g., synapsin I and Munc 18) have been shown to be a target of the p35/Cdk activity *(33,34)*.

Cdk5 is also involved in the regulation of the *N*-methyl-D-aspartate (NMDA) class of glutamate receptors *(35)*. NMDA receptors are essential for synaptic transmission and are critical for learning and memory. NMDA receptors are multimeric complexes formed from the receptor subunit NR1 and various splice variants of the modulatory NR2 subunit *(36)*. NMDA receptors consist of heteromeric NR1/NR2 complexes. Phosphorylation by serine/threonine and tyrosine kinases regulates the activation of the NMDA receptors (Fig. 4). Phosphorylation of the NR2A modulatory subunit at Ser-1232 in vitro and in intact cells by Cdk5 kinase was recently shown *(35)*. This modification was inhibited by ROSC, a Cdk inhibitor. Moreover, inhibition of Cdk5 prevented induction of long-term synaptic plasticity in CA1 pyramidal cells of rats, indicating that Cdk5 plays a crucial role in synaptic transmission through phosphorylation of the components of NMDA receptors.

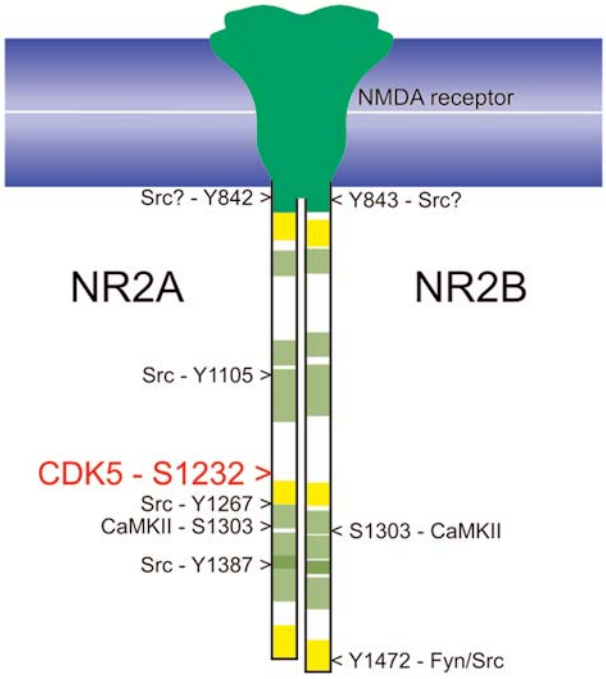

Fig. 4. Cdk5-mediated phosphorylation of the NMDA receptor regulates its activity. Adapted from ref. *35*.

The modification of its physiological substrates appears to be involved in proper cytoskeletal organization, neuronal migration, axonal transport, and neurite outgrowth *(37)*.

Abnormal Regulation of Cdk5 Activity Promotes Neurodegeneration

Cdk5 is required for normal development of the CNS, and its activation/de-activation cycles must be very tightly regulated. Short-lived p35 protein tightly controls the Cdk5 kinase activity. On the other hand, p25 (a truncated form of p35) is stable because of the lack of motifs for ubiquitylation, and it exhibits an inappropriate intracellular localization. Unlike p35, p25 is localized in the nuclear and perinuclear region (as revealed by immunostaining) *(15)*. By subcellular fractionation, p25 is enriched in the cytosolic fraction *(15)*. Plasma membrane anchoring of p35 appears to be mediated by its myristoylation within the NH_2-terminus. Because p25 lacks the conserved myristoylation signal, it is unable to target the plasma membrane.

High levels of p25 were detected in the brains of patients with Alzheimer's disease. Binding of p25 to Cdk5 constitutively activated Cdk5, changed its cellular distribution, and altered its substrate specificity. Activated p25/Cdk5 hyperphosphorylated τ protein in vivo, thus reducing its binding to microtubules (Fig. 5). This resulted in cytoskeletal disruption and induced apoptosis, suggesting that accumulation of p25 may be involved in the pathogenesis of cytoskeletal abnormalities and neuronal apoptosis occurring in neurodegenerative disorders.

The p53 Tumor Suppressor: Expression and Regulation of its Biological Activity

p53 phosphoprotein, the product of a tumor suppressor gene, is a transcription factor that regulates the expression of numerous downstream genes that possess a p53-specific consensus sequence in their promoters. Most p53-dependent genes are involved in the regulation of cell cycle progression, DNA repair, and apoptosis. Considering this key biological function of p53 protein, its cellular level must be tightly controlled. Therefore, in normal unstressed cells, p53 protein is maintained at low concentrations, primarily because of the regulative action of mouse double minute (mdm)-2 or human double minute-2 protein, its downstream transcriptional target.

Mdm-2 affects p53 in two ways: by regulation of its activity and by influencing its intracellular concentration. First, if upregulated, it inhibits the transcriptional activity of the p53 protein. Second, mdm-2 that possesses intrinsic E3 ubiquitin ligase activity targets p53 for polyubiquitylation and sequential proteolytic degradation in the proteasomes. This funda-

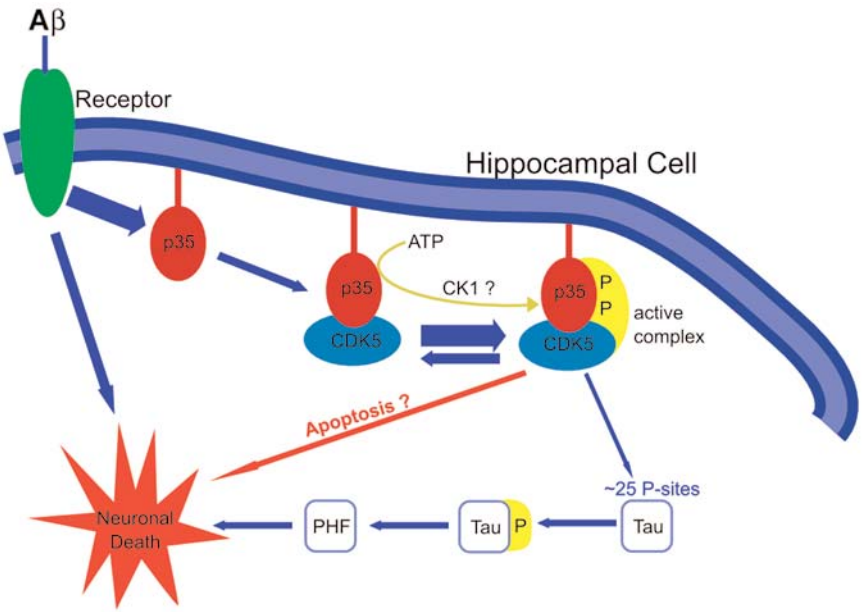

Fig. 5. Phosphorylation of τ-protein by activated Cdk5/p35 complexes.

mental role of mdm-2 in the regulation of p53 function is depicted by the finding that inactivation of mdm-2 by gene disruption is lethal and that embryos die very early because of cell cycle blockade and an enhanced apoptosis rate induced by upregulation of p53 protein. The autoregulatory feedback loop established between p53 and mdm-2, in which p53 induces expression of its own antagonist, can be abrogated by human alternative reading frame p14 protein ($p14^{ARF}$). $p14^{ARF}$, a product of the *INK4a* gene generated by alternative splicing, binds to mdm-2; through this interaction, $p14^{ARF}$ prevents mdm-2-mediated degradation of the p53 protein. However, in response to various cellular and environmental stress stimuli, p53 escapes from mdm-2 control, and its cellular level increases, inducing either transient cell cycle arrest or apoptosis. Therefore, wild-type p53 is a very important guardian of genomic stability and efficiently prevents development of cancer. Unfortunately, mutations of the *p53* gene, or inactivation of the p53 protein by its premature degradation or inappropriate intracellular localization, contribute to the development of neoplasia.

However, terminally differentiated cells and cells that undergo replicative senescence frequently express elevated p53 levels and exhibit enhanced wild-type p53 activity.

Upregulation and transcriptional activation of p53 protein leads to elevation of cell cycle inhibitors (e.g., $p21^{waf1}$) and blocks the progression of the cell cycle, providing necessary time for the cell to repair genomic damage before entering the critical stages of DNA synthesis and mitosis *(38)*. However, in tissues in which the stressors generate severe and irreparable damage, p53 can initiate apoptosis, thus eliminating damaged cells *(39)*.

Alternatively, wild-type p53 may mediate a terminal cell cycle arrest known as senescence *(40–43)*. Senescence observed in cultured cells is irreversible and is accompanied by an enhanced p53 activity.

p53 Expression and Distribution in Neuronal Tissues

In neuronal tissues, the elevated expression and activity of p53 is a prerequisite for the

induction of developmental apoptosis. Therefore, it is worth noting that the truncated p53 family member ΔN-p73 displays anti-apoptotic properties in developing neurons *(44)*. p53 is responsible for the induction of apoptosis in neurons that are not able to compete successfully for the limited amount of NGF and, therefore, have to be removed from the developing brain. A tight regulation of the balance between pro- and anti-apoptotic factors is extremely important for the appropriate development of the CNS and the peripheral nervous system. This can also be concluded from the defects observed in knockout mice that are missing one of the components of this fine-tuned developmental surveillance system *(45–50)*. In addition to its function in eliminating a subset of immature neuronal cells, p53 participates in the removal of dispensable or damaged mature cells in the neural tissues *(51,52)*. However, the dependence of apoptosis on p53 is not as pronounced in mature cells as it is in developing neuronal cells *(53)*.

Interestingly, researchers recently described that Notch receptors execute an important function in the activation of p53 in early neural progenitor cells but not in postmitotic neurons *(53)*. Notch receptors play a role in cell–cell interactions *(54)* and can be activated by binding ligands located on adjacent cells. This is followed by proteolytic processing through a disintegrin and metalloprotease/tumor necrosis factor α-converting enzyme family metalloproteases *(55, 56)* and then by a presenilin-dependent protease. These specific proteolytic steps result in the generation of the Notch intracellular domain *(57)* that translocates into the nucleus and participates in the transcriptional activation of its targets. Interestingly, upregulation of Notch activity leads to elevated p53 levels in the nucleus of neural progenitor cells, and the effect of Notch signaling is entirely suppressed in $p53^{-/-}$.

Note that the effect of Notch activation depends on the cell type and developmental stage. Studies have shown that Notch drives neural progenitor cells into a quiescent, nondifferentiating state, whereas it induces growth of glia cells at a later developmental phase *(58)*. In the immune system, the action of Notch is ambiguous because it is able to induce apoptosis in B-cells *(59)*, whereas it inhibits programmed cell death in T-cells *(60)*. In the case of neural progenitor cells, however, convincing data have shown that in mice conditionally overexpressing the Notch intracellular domain, extensive apoptosis occurs that depends on the presence of p53 protein *(53)*. The authors also showed that the brain—particularly the cerebral cortex of mice overexpressing the Notch intracellular domain—was significantly smaller than in control mice.

In the context of pathological situations in the brain, the function of p53 is not as clearly defined as in the development of neuronal cells. In the case of ischemia, caspases and apoptosis-inducing factor (AIF) appear to be the major players. The involvement of caspases was found to be necessary for the subsequent execution of apoptosis *(61)*. On the other hand, excessive poly(ADP-ribosyl)polymerase (PARP)-1 activation that results in AIF release from mitochondria and its subsequent nuclear translocation indicates existence of an additional, caspase-independent pathway of programmed cell death *(62,63)*. Upstream of the caspases and AIF, the transcription factors nuclear factor-κB, p53, E2F1, and others appear to be involved in the induction of apoptosis *(61)*.

Following mild ischemia, caspase-3 activation and other hallmarks of apoptosis can be observed in the brain hippocampus and caudate-putamen *(64–67)*. Apoptotic neuron death appears to be more prominent following transient ischemia than after permanent ischemia, and in the case of focal ischemia, there seems to be more apoptosis in the penumbra than in the core, where the predominant cell death mode becomes necrosis *(68–71)*.

In postmortem samples of the brain of patients with Alzheimer's disease, an elevated p53 immunoreactivity was found in the frontal and temporal lobe *(72)*. Immunohistochemical analysis showed signs of apoptosis and an increased p53 level in overlapping populations of cortical neurons as well as cortical and white matter glial cells in regions that were damaged by the neurodegeneration. The authors also

described colocalization of dystrophic p53-immunoreactive axons with amyloid-β (Aβ) immunoreactive plaques. This finding supports the assumption that Aβ deposits are associated with local neuritic degeneration. The authors have also suggested that apoptosis in glia cells may be an additional mechanism for loss of synaptic function in axons of patients with Alzheimer's disease *(72)*.

Interestingly, the level of p53 protein was also higher in the brain of transgenic mice that served as a model for Alzheimer's disease *(73)*. Another group *(74)* found elevated p53 immunoreactivity restricted to glia cells in the brains of deceased patients with Alzheimer's disease. They assumed that apoptosis depends on p53 in glia cells, whereas there might be p53-independent pathways that induce the apoptotic process in neurons. Immunoreactivity for p53 and increased levels of p53 messenger RNA (mRNA) were also found in aged human and Wistar rat brain *(71,72)*. These findings were confirmed in aged brains of Aprague-Dawley rats. The protein was detected in different types of neurons of the hippocampal CA1 layer, but not in the CA2 or CA3 layers, in middle-sized, multipolar, or fusiform neurons of the septal region or in the neurons of the cerebellum *(75)*. Because the pattern of mRNA *in situ* hybridization was consistent with the protein expression profile, the regulation of the p53 protein level appeared to occur at the transcriptional level rather than through protein translation or stabilization. In sharp contrast to aged brain, in normal adult brain, p53 protein could not be detected in any region *(75)*. Hippocampal pyramidal and cerebellar Purkinje neurons are among the largest and most vulnerable neurons regarding ischemia and hypoxia, respectively *(76,77)*. The authors concluded that upregulation and activation of p53 may be responsible for the loss of Purkinje cells during aging. This could be caused by compromised blood flow in the aged tissue.

p53-Induced Apoptosis

Apoptosis is a genetically programmed process of cellular suicide that is widely observed in organisms *(78,79)*. It can be triggered by a variety of stimuli, including various physiological processes, stressful conditions, and toxic insults. Apoptosis is characterized by a specific pattern of sequentially occurring morphological features that are accompanied by biochemical changes. The aim of apoptosis is to eliminate overproduced or severely damaged cells from tissues and organs, thus promoting development and terminal differentiation or preventing malignant transformation. Apoptosis-induced elimination of cells is based on the stepwise degradation of the chromosomal DNA and proteolytic cleavage of numerous cellular proteins; it is also based on the externalization of phosphatidylserine residues at the cell surface. The latter facilitates recognition of apoptotic cells by phagocytes. Apoptosis can be triggered by different mechanisms that are referred to as the intrinsic, extrinsic, or endoplasmatic reticulum pathways. Mitochondrial dysfunction bridges the distinct pathways.

The induction of apoptosis is central to the tumor-suppressing activity of p53 protein. Overexpression of p53 is sufficient to trigger apoptosis in malignant *(39)* and normal developing cells (e.g., postmitotic neurons *[51,80]*) and overexpression of p53 also induces apoptosis under some pathological situations *(81)*. Although p53-independent apoptosis also occurs, the crucial role of p53 in the regulation of apoptosis is reflected by the fact that p53 knockout mice exhibit reduced tissue and organ damage after exposure to various insults *(82)* as well as reduced brain damage after stroke *(52,83)*. The stimulated p53 protein directly enhances the activity of distinct pro-apoptotic genes such as *Bax, Apaf-1, Peg3, PUMA, Noxa (84)*, p53 apoptosis effector related to PMP-22 (*Perp*) *(85)*, and *caspase-9* and is also able to repress different genes *(86–88)*, including the anti-apoptotic gene *Bcl-2 (89)*. Because wild-type 53 is able to regulate pro-apoptotic proteins involved in the early and late phases of apoptosis, wild-type p53 is assumed to promote the execution of this process at different stages.

p53 protein possesses two distinct transactivation (TA) domains, and each can indepen-

dently activate p53 target genes. Studies have shown that the intact first activation domain (AD1) is indispensable for driving apoptosis following neuronal injury *(90)*. Mutations in the second activation domain (AD2) lead to a reduction in apoptotic activity *(91)*. The induction of the pro-apoptotic BH3-only proteins PUMA and Noxa exhibits a differential regulation by the two TA domains *(90)*. Whereas Noxa can be induced by either activation domain, induction of PUMA requires both activation domains to be intact. Conversely to Noxa, the upregulation of PUMA alone is sufficient to induce neuronal apoptosis *(90)*. The BH3-only members of the Bcl-2 family of proteins represent a unique group in which members solely share a common nine-amino acid motif with other family members within the BH3 domain *(92,93)*. Recent evidence strongly supports the assumption that unlike Noxa, PUMA is a potent inducer of neuronal cell death *(90)*. In one study, p53-mediated induction of PUMA strongly correlated with cell death, and ectopic expression of PUMA was sufficient *per se* to induce apoptosis *(90)*. The observation that PUMA-deficient neurons were relatively resistant to apoptosis induced by overexpression of p53 compared to neurons of their wild-type littermates is consistent with this line of evidence *(90)*. Interestingly, PUMA may also be regulated by other factors, such as p73 *(94)* and E2F *(95)*.

Although most of the effects of wild-type p53 are attributable to its function as a transcription factor, some reports have suggested that p53 is capable of cell death induction in a transcriptionally independent manner *(96–98)*. The importance of the proline-rich motif of p53 has been suggested to be essential for induction of apoptosis *(99)*. The mitochondrial localization of p53 mutants defective for transcription also facilitates apoptosis through dissipation of the mitochondrial potential *(100)*.

The molecular mechanism by which wild-type p53 initiates apoptosis independently of transcription appears to involve the mitochondrial targeting of p53 and its direct binding to one or more anti-apoptotic mitochondrial proteins (e.g., Bcl-X_L). The p53-mediated sequestration of anti-apoptotic proteins prevents their suppressive effect on outer membrane pore formation by Bax or Bak and, consequently, prevents cytochrome-*c* release *(101,102)*.

One study recently reported a direct activation of Bax by p53 localized in the cytosol *(103)*. A cytosolic localization of endogenous wild-type p53 was both necessary and sufficient for apoptosis. p53 activated Bax and released both pro-apoptotic multidomain proteins and BH3-only proteins *(103)*. A TA-deficient p53 exhibited the same effect *(103)*. The transcription-independent activation of Bax by p53 protein occurred with similar kinetics and to a comparable extent to that generated by activated Bid *(103)*. These observations substantiate previous results showing that p53 can function at the mitochondrial level *(96,102,104)*.

Although in neurons the most important molecular components in p53-dependent cell death are Bax and PUMA, involvement of other downstream molecules has been reported *(105,106)*. Studies have demonstrated that p53 is required for caspase activation only in response to some forms of neuronal injury *(106)*. These findings implicate that p53 mediates neuronal apoptosis after injury via two pathways: a caspase-dependent pathway and a caspase-independent pathway. The conditional contribution of caspase activation to cell death depended on the state of neuronal maturation. Therefore, the relative importance of caspase activation in neurons appears to depend on the developmental status of the cell and the type of death stimulus.

Recently, a new p53-dependent pro-apoptotic gene, p53-apoptosis inducing protein-1 (*p53AIP-1*), was identified *(84)*. *p53AIP-1*, a component of the mitochondrial membrane, has been found to be regulated by p53 protein in a highly specific manner *(107)*. Only wild-type p53 protein phosphorylated at Ser46 was trancriptionally competent to induce p53AIP-1 protein *(24,25)*. Upon severe DNA damage, Ser46 on p53 was shown to be phosphorylated, resulting in the induction of p53AIP-1. This was followed by depolarization of the mito-

chondrial membrane, which in turn led to the release of distinct mitochondrial proteins such as cytochrome-*c* and AIF *(84,107)*. Ectopically expressed p53AIP-1 protein, which was localized in mitochondria, led to apoptosis through dissipation of the mitochondrial potential *(84,108)*.

Recently, transcriptional activation of the *p53AIP1* gene was described in human MCF-7 breast cancer cells *(109)*. The transcriptional upregulation of the *p53AIP1* gene occurred in parental MCF-7 cells but not in cells reconstituted with caspase-3 after treatment with ROSC, a Cdk inhibitor *(109)*. It was consistent with the ROSC-induced phosphorylation of p53 at Ser46 *(109)* in the former and preceded the depolarization of mitochondria. The *p53AIP1* gene generates three transcripts (α, β, and γ) by alternative splicing encoding peptides of 124, 86, and 108 amino acids, respectively. Because p53AIP1α and p53AIP1β are localized in the mitochondria, they are proposed as mediators of the mitochondrial membrane potential. The upregulation of *p53AIP1* gene by ROSC in MCF-7 cancer cells *(109)* appears important because of the potential therapeutic application of Cdk inhibitors in the treatment of neurodegenerative diseases.

Cdk5–p53 Interaction

Although activation of Cdk5 was observed during neuronal apoptosis, there was a long period during which the mechanism by which Cdk5 may promote apoptosis was not well-understood. The interaction between Cdk5 and the p53 tumor suppressor was recently investigated. During apoptosis induced in PC12 cells by NGF withdrawal, levels of p53 and Cdk5 concomitantly increased *(110)*. Research has shown that the p25/Cdk5 complex phosphorylated recombinant p53 protein in vitro as well as in cells that only transiently expressed the p25/Cdk5 complex *(110)*. Cdk5-mediated phosphorylation of the p53 protein was efficiently inhibited in the latter by ROSC, a selective inhibitor of Cdks. Transient expression of p25/Cdk5 resulted in an upregulation of p53 that was functionally active. Distinct p53 downstream genes such as $p21^{waf1}$ and Bax were elevated *(110)*. These data implicate that Cdk5 may facilitate apoptosis by phosphorylation and activation of the p53 protein. Constitutive activation of Cdk5 by p25 can be proposed to excessively activate p53 and may play a role in the neurodegenerative processes of Alzheimer's disease.

Crosstalk Among the p53 Family Members

The p53 family consists of three family members—namely, p53, p63, and p73. Each family member is expressed in several isoforms generated via alternative splicing as well as through the usage of alternative promoters. Generally, the full-length or TA isoforms of the proteins are pro-apoptotic and, in some cases, are negative cell cycle regulators. The truncated or ΔN isoforms counteract these actions; therefore, their influence on the cells is anti-apoptotic. The ΔN isoforms are generated through the use of an alternative promoter located downstream of the first promoter that gives rise to the full-length isoforms. The expression of the different isoforms varies strongly among various tissues and cell types, and the ΔN forms act in a dominant-negative way by inhibiting their full-length counterparts.

In normal cells, p53 is almost exclusively found as the full-length protein, whereas the truncated version is found only in certain tissues *(111)*. On the other hand, the p63 and p73 proteins are expressed as several full-length and truncated proteins. In the case of p63, at least three full-length (α, β, γ) and three truncated versions of the protein have been described *(112)*. For p73, at least six full-length (α, β, γ, δ, ε, ζ) and several truncated variants are expressed *(113–116)*. Notably, in a given cell type, only some isoforms of the proteins are usually expressed in significant amounts.

Interestingly, p63 *(112)* and p73 *(117,118)* have only recently been found, although p53 has been the focus of intense research for decades. This may partly result from the fact that p53 is absent or mutated in many types of cancer *(119)* and it binds to several viral oncoproteins with which it could be easily copurified *(120–122)*. The TA forms of p63 and p73 are capable of inducing the expression of some p53 targets by transcriptional activation (e.g., Bax, PERP, and Noxa), whereas p53 is the only family member capable of activating others (e.g., mdm-2 and p21) *(123,124)*. Targets also inducible by p63, p73, or both generally comprise proteins involved in apoptosis, whereas noninducible genes include factors connected to cell cycle arrest *(123)*. Most importantly, some genes can be expressed only when p53 and at least one full-length variant of p63 or p73 bind to the promoter *(123)*. Structurally, the three family members show a high degree of homology, and similarly to the founding member p53, each variant comprises an N-terminal TA domain, a central DNA-binding domain, and a C-terminal oligomerization domain, which also harbors a nuclear localization signal *(125)*. p63 and p73 can also be activated by DNA-damaging agents *(126)*, and mouse embryo fibroblasts lacking either p63 or p73 are more resistant to chemotherapy than wild-type cells *(123)*. Mouse cells that lack p63 and p73 are similarly resistant to chemotherapy, which is comparable to p53 knockout cells *(123)*.

The results from experiments using p63 or p73 knockout mice or cells from these mice have been difficult to interpret, because all TA and ΔN isoforms of the proteins were knocked out simultanously in the classical models. However, p73 knockout mice *(127)* have been shown to display neural abnormalities and have problems with their immunological response, and they die within the first weeks after birth. p63 knockout mice show even stronger developmental defects and have missing or truncated fore- and hindlimbs as well as several other developmental deficits *(128,129)*.

The significance and counteracting nature of the truncated and full-length forms has only recently been clarified. In the future, the employment of the small interfering RNA approach and other techniques capable of inhibiting only certain isoforms of the p53 family members should further elucidate the action of specific variants of the respective proteins. The ability of the p53 family members to act as both pro-apoptotic and pro-survival factors with the sophisticated interaction among them allows them to keep a fine-tuned balance and to integrate a wide variety of signals. This places them in an ideal position to decide the fate of a cell depending on the circumstances, thereby protecting the whole organism from deleterious effects that could arise from severely damaged cells.

Neuronal Apoptosis After Transient Brain Ischemia

The elimination of both progenitor cells and postmitotic neurons overproduced during development of the nervous system occurs during two major waves of cell death. The first occurs in the ventricular and subventricular regions of the developing nervous system. The second period of cell elimination occurs as neurons migrate to their final destination. It is widely accepted that during normal development of vertebrates, apoptosis is the mechanism of cell death operative that regulates the differentiation, outgrowth, and coordination of the appropriate stoichiometric ratio of the two main neuronal cell populations: cerebellar granule cells and Purkinje cells. The developmental apoptosis of cerebellar granule cells does not depend on p53 status and occurs similarly in wild-type and $p53^{-/-}$ mice *(130)*. However, p53 appears essential for ionizing-radiation-induced apoptosis in developing brain. Ionizing radiation induced extensive cerebellar granule cell death in 1-wk-old wild-type mice. However, in $p53^{-/-}$ mice, cerebellar granule cells did not undergo apoptosis in response to X-ray damage, and apoptosis was delayed in mice heterozygous for the *p53* allele, indicating an intermediate response *(130)*.

The elevated rate of cell death in developing brain is reflected by the fact that some apoptotic factors are more highly expressed in the brain of developing animals compared to those in adult animals. The determination of the caspase-activated DNase (CAD/DFF40) mRNA in cerebellum of developing rat brains revealed marked differences between developing and adult rats. High levels of expression were detected in 17-d-old embryos and in 1- and 2-wk-old rats, whereas the level was decreased approximately fivefold in the adult rats *(64)*. The elevated expression of CAD/DFF40 in cerebellum of developing rats reflects the massive neuronal apoptosis that occurs in this region during brain development.

Under pathological conditions such as animal models of cerebral ischemia, either necrosis or apoptosis occur *(131,132)*.

Considering that in brain ischemia, the morphological studies indicated necrotic cell death, whereas the biochemical studies showed apoptotic markers, in some instances, the exact discrimination between both cell death processes is very difficult.

Following a short-term severe oxygen deprivation, necrotic cell death is primarily induced in neuronal cells *(132)*. On the other hand, transient focal or global ischemia results in apoptosis *(131)*. Cell death is selective and delayed, appearing 48 to 72 h after the initial insult *(131,133,134)*. Morphological changes that are characteristic of apoptosis *(135)* are induced mainly in the hippocampal CA1 region, in scattered neurons in the cerebral cortex, and in the striatum.

Hypoxia elicits various adaptive responses at different organizational levels of the body. A physiological response to oxygen deprivation requires the existence of an O_2 sensor linked to a signal transduction system, which in turn induces a functional response *(136)*. Although numerous studies exist on the signaling systems activated by hypoxia, there is no consensus regarding the nature of the underlying O_2 sensor or whether multiple sensors exist independently. More recent data indicate that mitochondria may function as oxygen sensors by increasing their generation of reactive oxygen species after hypoxia *(137)*. This implicates the mitochondria in the initiation of signaling cascades involved in the adaptive responses to hypoxia that simultaneously participate in the control of cell death pathways.

Reduction of the normal cellular O_2 concentration in animal tissues results in stabilization and activation of hypoxia-inducible factor (HIF)-1 *(138)*. Upregulated HIF-1 induces transcription of genes whose products (e.g., glycolytic enzymes or glucose transporters) increase O_2 delivery and/or provide metabolic adaptation to reduced oxygen concentration. Interestingly, HIF-1 also induces wild-type p53 protein *(139)*, suggesting that it is involved in hypoxia-induced apoptosis in vivo. Hypoxia-induced p53 appears to be transcriptionally inactive. Although the role of hypoxic induction of p53 has not been fully elucidated, some lines of evidence indicate that p53 specifically binds to p300 (leading to an interference in HIF-1/p300 TA) and that this finally mediates repression of HIF-1 activity *(140)*.

The activation of caspase-3 in brain after cerebral ischemia has been detected in several animal models *(141)* as well as in human autopsy brain tissue *(142)*. Beginning 8 to 72 h after ischemia, activated caspase-3 and caspase-activated DNase were detected in the degenerating hippocampal CA1 neurons. Both proteins translocated to the nuclei 24 h after initial insult. The accumulation of caspase-activated DNase in the nuclei coincided with the cleavage of cytosolic inhibitor of caspase-activated DNase and preceded the internucleosomal DNA fragmentation in hippocampal neurons within 48 h *(64)*. The activation of nuclease and DNA cleavage was accompanied by nuclear changes in ischemic neurons that were characteristic for apoptosis. The activation of caspase-3 and caspase-activated DNase was also detected in neuronal cultures under ischemia-like conditions and was inhibited by z-DEVD-fmk peptide, which encompasses the tetrapeptide motif specific for the caspase-3 cleavage site.

p53 can repress the HIF-1 activity in a manner that does not require the trans-activating

function of p53 *(140)*. A significantly greater amount of p53 is necessary to inhibit HIF-1 than that which is required to transactivate p53 downstream genes or cause gene arrest because p53 must bind stoichiometrically to HIF-1/p300 complexes to inhibit HIF-1. The physiological relevance of this phenomenon is reflected by two observations. First, the level of p53 that must be reached to repress HIF-1 is equivalent to that at which PARP-1 cleavage becomes detectable. Second, this level can be reached in vivo following exposure to several commonly used anti-cancer drugs.

The involvement of p53 in neuronal cell death is well established. However, its role remains controversial. p53 levels have been found to be increased after stroke *(143)*. The upregulated p53 appears to be transcriptionally inactive, and in this case, it has a beneficial role: the inactivation or repression of HIF-1. On the other hand, the size of ischemic regions in the brains of $p53^{-/-}$ mice has been reduced *(52,144)*.

Apoptosis in Neuronal Cells Controlled by the p53 Family Members

In recent years, the interaction of the p53 family members p53, p63, and p73 has received much attention; therefore, we have gained insight into the pro- and anti-apoptotic pathways in neuronal cells. In neurons, apoptosis is of key importance in early development, after traumatic injury, and in neurodegenerative conditions. During the developmental period of programmed cell death in the CNS and the peripheral nervous system, the appropriate number of neuronal cells is reached after elimination of cells that are unable to acquire a pro-survival phenotype. This developmental step is necessary to match the number of neurons to the size of their targets *(145)*. By default, all neurons are destined to die; only cells capable of receiving enough growth factors from their target cells—especially NGF—are permitted to evade apoptosis. Usually, up to one-half of all neurons in a developing organism are eliminated via this pathway *(145)*. After a time window, during which the neuronal cells are extremely prone to apoptosis, changes in the cells occur that make them relatively insensitive to programmed cell death.

Interestingly, p53 and its family members are the key proteins in a checkpoint in developing as well as in mature neurons *(146,147)*. p53 has long been known for its pro-apoptotic role in neurons *(126)*, but only recently has the crucial pro-survival function of the truncated variants of p63 and p73 been described in cells of the CNS and the peripheral nervous system *(148)*. The lack of a clear developmental defect of p53 knockout mice led to the false conclusion that the protein was dispensable. After more thorough investigations, research found that an imbalance existed in the ratio of male and female knockout mice and that a large number of female embryos died *in utero*. This resulted from a midbrain exencephaly caused by an overproduction of neural tissue that resulted in a failure of neural tube closure *(45,49)*. Interestingly, similar developmental problems were found in mice in which Apaf-1 *(46,50)*, caspase-3 *(48)*, or caspase-9 *(47)* were knocked out. These proteins all play a crucial role in the mitochondrial death pathway. Until the other family members were found capable of partially compensating for the loss of p53 under certain circumstances, researchers had difficulty explaining the reason that the p53 knockout phenotype was not more severe.

Interestingly, the predominant isoforms of p73 in neurons are the anti-apoptotic ΔN isoforms, and loss of p73 leads to enhanced neuronal apoptosis *(44,149)*. It has also been shown that the truncated p73 isoforms are necessary for the long-term maintenance of CNS and peripheral nervous system neurons. Sympathetic neurons survive the phase of developmental death when they are able to sequester sufficient amounts of target-derived NGF. In that case, NGF activates a survival signal through the receptor tyrosine kinase A/NGF receptor. This receptor mediates its effects by activating the phosphatidylinositol 3–kinase–

Akt pathway. The p75 neurotrophin receptor, a functionally antagonistic receptor to tyrosine kinase A, mediates its pro-apoptotic function through signaling via the c-Jun N-terminal protein kinase–p53–Bax pathway *(150)*.

A recent report has provided more insight into the function of p53 and the truncated forms of p73 in sympathetic neurons *(151)*. This work showed that ΔNp73 is the crucial protein that ensures the survival of sympathetic neurons, and that the protein exerts its function via p53-dependent and -independent mechanisms. It was reported that $p73^{-/-}$ mice died within 2 wk after birth and that they showed a strongly decreased sympathetic neuron number. On the other hand, $p53^{-/-}$ and also $p53^{+/-}$ mice had an increased neuron number because of a reduced apoptosis rate, demonstrating the antagonistic role of the two family members. Most importantly, the $p73^{-/-}$ mice showed only a partial rescue from a decreased number of neurons when p53 was also knocked out *(151)*, pointing to p53-independent mechanisms in the action of ΔNp73. Additionally, the smaller neuron size found in p73 knockout mice could not be rescued in p73/p53 double-knockout mice *(151)*. In their report, Lee and co-workers *(151)* demonstrated that ΔNp73 could bind to c-Jun N-terminal protein kinase, thereby inhibiting the signaling pathway leading to the mitochondrial apoptotic transition in a p53-independent manner. Additionally, the authors claimed that cultured $p63^{-/-}$ sympathetic neurons displayed a decrease in apoptosis following withdrawal of NGF that was even more pronounced than the descrease in $p53^{-/-}$ neurons. Obviously, the life vs death decision in sympathetic neurons depends on the balance between the pro-survival ΔNp73/ΔNp63 isoforms and the pro-apoptotic, full-length p53/TAp63/TAp73 isoforms.

Pharmacological Neuroprotection After Ischemia

Considering the involvement of p53 protein in the induction and execution of apoptosis after transient cerebral ischemia, the inhibition of the p53 transcriptional activity could induce neuroprotection and reduce the long-term consequences of ischemia. The neuroprotective action of activated protein C has recently been described *(152)*. Activated protein C, a systemic anti-coagulant and anti-inflammatory factor, inhibited p53-mediated apoptosis in ischemic human brain endothelium.

Encouraged by these observations, Leker et al. *(143)* made attempts to block the p53-induced apoptosis in cerebral ischemia using pifithrin-α, an inhibitor of p53 *(143)*. Pifithrin-α has been shown to inhibit the transcriptional activity of p53 without affecting the level of p53 protein.

Novel derivatives of hexahydrobenzothioazole and hexahydrobenzoxazole were recently synthesized *(153)*. These compounds are p53 inactivators and were tested in a rodent model of stroke. They exhibited neuroprotective activity in models of transient and permanent focal ischemia. The infarct volume was reduced in animals pretreated with the drugs. Furthermore, these compounds also protected neuronal cells in in vitro and in vivo experiments against apoptosis induced by anti-cancer drugs such as etoposide and campthotecin *(153)*. However, is appears that specific inhibitors of PARP-1 represent the most promising neuroprotective agents after ischemia *(154)*.

Pharmacological Inhibitors of Cdks as Promising Drugs for the Therapy of Neurodegenerative Diseases

As previously mentioned, accumulating evidence shows that Cdk5, upregulated and constitutively activated in neurodegenerative disorders, causes hyperphosphorylation of τ and neurofilament proteins, leading to neuronal cell death. Therefore, the overactivation of Cdk5 plays a key role in their pathogenesis. The essential role of Cdk5 in neuronal cell death provides a rationale for the application of Cdks in the therapy of neurodegenerative

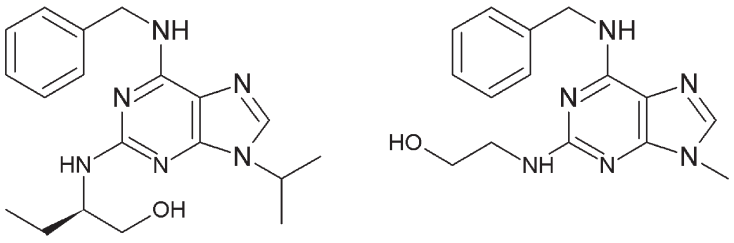

Fig. 6. Structure of the Cdk inhibitors roscovitine and olomoucine.

diseases such as Alzheimer's disease or amyotrophic lateral sclerosis *(155,156)*.

Recently, numerous specific and efficient inhibitors of Cdks have been developed *(157,158)*. Substituted purines and pyrimidines exhibit low direct cytotoxicity and are not mutagenic, whereas Cdk inhibitors with purine-based skeletons such as olomoucine or ROSC (Fig. 6) are structurally most closely related to adenosine triphosphate, whose binding they antagonize. ROSC inhibits different cyclic/Cdk complexes, and at very low concentrations, it exhibits a strong effect on Cdk2, Cdk1, and Cdk5. Considering its increased selectivity and the capacity to affect Cdk5, ROSC became a promising candidate for treatment of neurodegenerative diseases. ROSC and its enantiomer *R*-ROSC (manufacturer code CYC202 and Seliciclib) has been studied in completed phase I clinical trials in healthy volunteers as well as in patients with malignancies. The efficacy of ROSC is currently being examined in phase II trials in several clinical centers. The study in healthy volunteers was performed to gain a more detailed understanding of the pharmacokinetics of ROSC, and it showed that the drug is well-tolerated by patients at doses exceeding 800 mg twice daily for 5 d; it also illustrated occurrence of only mild and transient side effects *(159,160)*.

In primary cultures of rat hippocampal cells, the neurotoxic effect of Aβ protein was markedly diminished following inactivation of Cdk5 using antisense Cdk5 RNA or the pharmacological inhibitor butyrololactone I *(161)*. In in vivo studies, olomoucine and ROSC prevented apoptosis of central and peripheral neurons induced by withdrawal of neurotrophic support from CNS and peripheral nervous system neurons *(162)*. Because Cdk inhibitors such as ROSC or butyrololactone I *(163)* affect not only Cdk5 but also Cdk1 and Cdk2, newer more selective agents lacking unwanted cytostatic effects have recently been developed *(164,165)*.

Under experimental conditions, evidence indicates that inhibition of Cdk5 by indolilone A, a selective Cdk inhibitor, protects neuronal cells against cell death by apoptosis and necrosis *(165)*. The neuroprotective mechanisms of action of indolilone A have been studied exhaustively. This new drug prevented mitochondrial dysfunction *(165,166)*. The phosphoproteome and transcriptome analysis revealed that this compound promoted both neuronal survival and neurite outgrowth *(166,167)*.

Interestingly, new naturally occurring compounds that selectively target Cdk5 (e.g., flavonoids and alkaloids) have been characterized recently *(168,169)*. These new discoveries based on the design of neuroprotective compounds and advances in the testing of new and highly selective Cdk5 inhibitors are very promising and are essential for the development of a new and efficient therapy for Alzheimer's disease and similar pathological conditions that are the leading cause for senile dementia, which affects more than 4 million people worldwide.

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