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REGULATION OF CYCLIN-DEPENDENT KINASE 5 (CDK5) IN CELL DEATH

by

LIN LIN

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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Abstract

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Lin Lin

Advisor: Dr. Zahra Zakeri

Cell death plays an important role in development, homeostasis and pathology. This biological process is tightly controlled by the coordination of different cell death signaling pathways. Originally identified as a cyclin-dependent kinase (Cdk) due to its sequence homology to Cdc2, it has been shown that Cdk5 is activated during cell death in some situations. The aim of this study is to elucidate whether there is a general correlation between Cdk5 activation and cell death, then evaluate the regulation of Cdk5 activation during cell death and the relationship between Cdk5 activation and other signaling pathways during cell death.

By using different models in which various pathological cell deaths are induced, including rat lungs suffering from pneumonia caused by *streptococcus pneumoniae* type 25, mouse limbs infected with *Leismania major*, preterm lamb lungs after liquid or gas ventilation, and cyclophosphamide (CP)-treated mouse embryos, we have expanded the correlation between Cdk5 activation and cell death to a broader spectrum of systems. We therefore confidently conclude that there is a general Cdk5 activation during cell death. The finding that Cdk5 is the only Cdk that is associated with cell death in CP embryos further suggests the unique role of Cdk5 during cell death compared to other Cdks, which

are master regulators of cell cycle progression. Furthermore, in CP-treated mouse embryos, by comparing the regulation of Cdk5 at the level of transcription, translation and post-translation, we have demonstrated that Cdk5 is up-regulated at the post-translational level, i.e. kinase activity, during cell death. Additionally, this study for the first time has shown that Cdk/p25 activation is mediated by calpain activation in non-neuronal cell death. Finally, we have demonstrated that p53 and apoptosome/caspase-9/caspase-3 are not required for Cdk5/p25 activation during cell death.

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Chapter I. SIGNIFICANCE OF CELL DEATH

1. Importance of cell death in development

2. Importance of cell death in homeostasis

3. Importance of cell death in pathogenesis

Cell death is an integral part of the normal life of an organism. Although cell death was noticed as early as in 1842 in the observation of amphibian metamorphosis by Vogt (for review, see Ranganath & Nagashree, 2001), the study of it remained very limited until the early 1990s. Today, cell death has turned into one of the hottest areas of modern biology. It is clear that cell death plays a key role in development, homeostasis and pathogenesis. Since in this thesis we are going to discuss the regulation of a gene, *cdk5*, during cell death, we will first emphasize the importance of cell death in different aspects, i.e. development, homeostasis and pathology.

1. Importance of cell death in development

During development, cell death can be classified into three classical types: apoptosis, autophagy (also referred as lysosomal cell death), and necrosis (for review, see Zakeri & Lockshin, 2002). Apoptosis and autophagy are also termed as type I and II programmed cell death (PCD), respectively. PCD describes a type of cell death that leads to a cell's destruction following a genetic program in multicellular organisms, which will be discussed in detail in Chapter II. Like proliferation and differentiation, cell death, especially PCD, also plays a prominent role in normal development. It is essential for the removal of unwanted cells and is critical for both restricting cell numbers and for tissue patterning during development (Glucksmann, 1951; Coucouvanis et al, 1995; Chanoine & Hardy, 2003).

Cell death starts at a very early stage in development. For example, in mammalian embryogenesis, cell death is detected as early as in blastocysts (Hardy et al, 1989; for review, see Spanos et al, 2002). During development, cell death plays an

important role in the elimination of redundant cells. In *C. elegans*, death of 131 of the original 1090 cells leaves an adult with 959 somatic cells (Horvitz et al, 1982). Precise patterns of cell death are critical for the development of nervous and immune systems. During vertebrate brain development, due to limited amount of trophic factors such as nerve growth factor (NGF), the only neurons that survive are those supplied with NGF. Apoptosis induced by NGF withdrawal decides the number of developing neurons (Jacobson et al, 1997; Reis et al, 2002; Vyas et al, 2002). Twenty percent to 80% of the neurons originally produced are eliminated by cell death (Gordon, 1995), and more than 80% of ganglion cells in the cat retina die shortly after birth (Barres & Raff, 1999). In the human immune system, about 95% of developing B cells will die due to faulty gene rearrangement, anti-self receptor expression, or lack of stimulation, and immunological tolerance is achieved through highly controlled cell death (Osborne, 1998; Gercel-Taylor et al, 2002). Cell death is also a pivotal part of the development of other systems. In human females, oocyte apoptosis results in the loss of more than 80% of the original germ cells by the time of birth (for review, see Reynaud & Driancourt, 2000).

Cell death also plays a crucial role in tissue remodeling, indicated by the substantial levels of cell death during morphogenesis. It is in metamorphosis that cell death was originally noticed. Cell death destroys almost all larval tissues during metamorphosis of insects and amphibians (Fox, 1973). In *Drosophila*, cell death is induced in larval midgut by the steroid hormone ecdysone (Lee et al, 2002). In tadpoles, regression of the tail and destruction of the gills are attributed to tightly controlled cell death (Smith & Tata, 1976; Jeffery et al, 2002). Precisely regulated cell death contributes to the formation of different body parts and multiple organs of an organism. Cell death

sculpts the limbs of mouse and birds by removal of interdigital webs (Saunders & Fallon, 1967; Ballard & Holt, 1968; Zakeri & Ahuja, 1994; Zakeri et al, 1994; Hurle et al, 1996). In developing rat lens, apoptosis removes the nuclei in the process termed nuclear death, and leaves the rest of the cells remaining functional (Gao et al, 1997; Gupta et al, 2002; Wu et al, 2002). A recent study on the development of the rat lens vesicle in relation to apoptosis has further suggested that apoptosis plays a major role during the lens vesicle development. In this process of development of lens vesicle, apoptosis eliminates the cells between the surface ectoderm and the optic vesicle to help trigger invagination and facilitate separation from the ectoderm. Apoptosis also aids in the bowing of the optic vesicle during lens invagination (Mohamed & Amemiya, 2003). Apoptotic cell death helps shape the future inner ear structure, which starts from incubation day 5 in chick inner ear (Avallone et al, 2002). In cardiac morphogenesis, cell death is essential in generating the overall four-chambered architecture of the heart (Abdelwahid et al, 2002). The role of cell death in muscle development has been well studied. In moths, muscle cell death is required for correct muscle patterning (Fahrbach et al, 1994; Haas et al, 1995). In rat skeletal muscle, cell death persists during the first three postnatal weeks, suggesting an indispensable role for cell death in the development of skeletal muscle (Torres et al, 2002). Very recently, some researchers found that in *Xenopus* cell death destroys all the primary myotomal myofibers, which are replaced progressively by secondary "adult" multinucleated myofibers in the construction of muscles during development (Chanoine & Hardy, 2003).

Developmental cell death is a highly conserved evolutionary process that is also involved in plant development. In plants, cell death plays an important role in a variety

of aspects from fertilization through development as well as in response to environmental insults. For example, PCD is involved in xylogenesis, aerenchyma formation, petal senescence, endosperm development, hypersensitive response and various forms of abiotic stress (for review, see Hoeberichts & Woltering, 2002). Although cell death in plants sometimes resembles apoptosis in many facets, there is unique cell death found in plants that displays many functional vacuoles and depends on vacuolar lytic function (Fukuda, 2001; Kuriyama & Fukuda, 2002). Reactive oxygen species (ROS) have emerged as important signals in the activation of plant PCD. For instance, in soybean cells, PCD can be triggered by ROS (Solomon et al, 1999). Increasing evidence indicates that plant PCD proceeds through a cell death mechanism that is functionally conserved between animals and plants (for review, see Hoeberichts & Woltering, 2002).

Cell death process is tightly controlled by death machinery composed of different gene products. The most convincing evidence that cell death is needed for development comes from studies of animals in which different cell death related genes have been knocked out. Members of the caspase family are among the most important players in the cell death machinery. Since in this thesis we will evaluate the relationship between the caspase pathway and the regulation of Cdk5, caspases are to be discussed in much more detail in a later chapter. Briefly, caspases consist of initiator (such as caspase-2, -8, -9) and effector (including caspase-3, -6, -7) caspases, which mediate a proteolytic cascade during cell death.

Deletion of the key death effector caspase, caspase-3, in mice in most cases is embryonically lethal. If birth occurs, caspase-3-/- mice are born at a frequency lower than expected by Mendelian genetics, and are smaller than their wild type littermates, and

die at 1-3 weeks. Brain development in caspase-3-deficient mice is profoundly affected, exhibiting a variety of hyperplasias and disorganized cell deployment in the cerebral cortex, the hippocampus, and the striatum. Furthermore, pyknotic clusters at sites of major morphogenetic change during normal brain development are not observed in the mutant embryos, indicating decreased apoptosis in the absence of caspase-3 (Kuida et al, 1996). Deletion of caspase-9 yields a phenotype essentially identical to that of caspase-3 null mice (Hakem et al, 1998; Kuida et al, 1998). Apaf-1 (apopotosis protease-activating factor-1, the function of which is to activate caspase-9) deficient mice share similar and profound defects in the developing brain with caspase-3 and –9 knockouts, revealing a lower incidence of apoptotic cells in their hindbrains compared to wild type mice. In addition, Apaf-1 knockout mice exhibit delayed interdigital mesenchymal cell death and alterations in the development of lens and retina (Colussi and Kumar, 1999). Mice carrying a null mutation in caspase-2 develop normally and do not show an overt phenotype. The most prominent feature of caspase-2-deficient mice is an inhibition of female germ cell death, which results in a significantly higher number of primordial follicles in knockout mice compared to wild type counterparts (Bergeron et al, 1998). Null mutations in caspase-8 result in embryonic lethality in mice, which have developmental abnormalities in cardiac tissue characterized by thin ventricular myocardium. In addition, the caspase-8 mutant mice display hyperaemia in the abdomen and other blood vessels with extensive erythrocytosis in the liver (Varfolomeev et al, 1998; Yeh et al, 1998). Caspase-7 knockout mouse embryos die during early embryogenesis with severe heart abnormalities (Zheng et al, 1999). Therefore, these

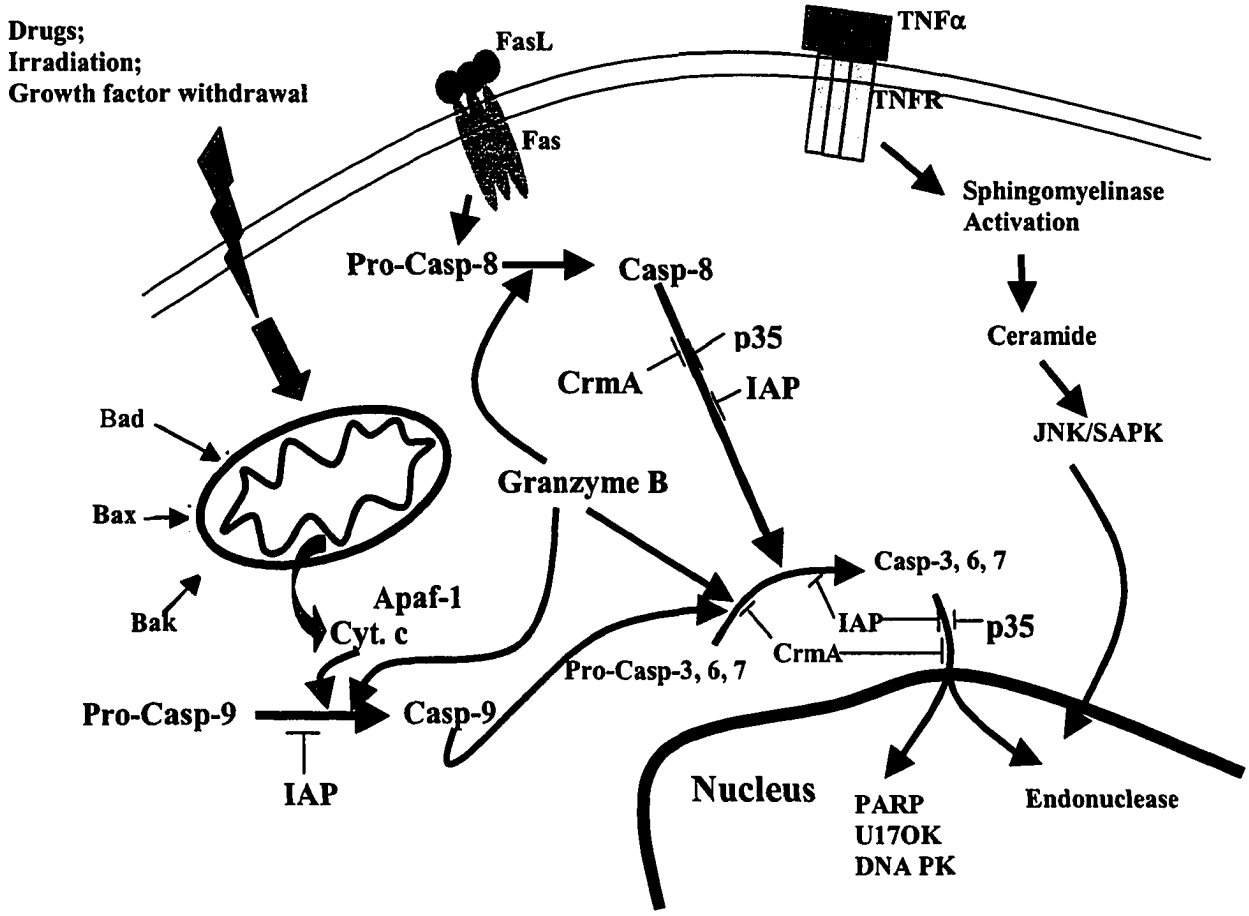
caspase knockout studies support the argument that caspase-mediated cell death plays a major role in different cell death pathways involved in development.

The bcl-2 family is another group of key players in cell death. The family is composed of both anti-apoptotic and pro-apoptotic members. Members such as bcl-2 and bcl-X_L inhibit apoptosis, whereas others promote apoptosis, like bid, bax, and bad. It has been found that the protein-protein interaction between bcl-2 members regulates cell death. Mouse embryos lacking the anti-apoptotic factor bcl-2 have a significantly greater number of trigeminal neurons undergoing apoptosis at day 14 and contain dramatically fewer neurons in trigeminal ganglia by day 16 and 18 (Pinon et al, 1997). Mice deficient in another anti-apoptotic bcl-2 family member, bcl-X_L, die at embryonic day 13 with excess apoptosis in developing brain, spinal cord and dorsal root ganglia (Motoyama et al, 1995).

Together, studies of these mutant mice have unearthed evidence for the essential role cell death mediated by caspases and or bcl-2 members plays in normal development of the central nervous system (CNS) and other tissues such as heart and liver. However, as shown in diagram 1 below, there are many important players of cell death other than caspases and bcl-2 members, which may also play crucial roles in development. For example, perforin/granzyme-induced apoptosis is the main pathway used by cytotoxic lymphocytes to eliminate virus infected or transformed cells (Trapani & Smyth, 2002). Furthermore, the mutant mice study has suggested that the level of cell death is critical for normal development and has to be highly controlled during development. Abnormal levels of cell death will lead to abnormalities in development, such as teratogenesis caused by teratogen-induced cell death in developing embryos. Studies have shown that

teratogens, such as 4-hydroperoxycyclophosphamide and staurosporine, induce apoptosis through activation of the mitochondrial pathway in early postimplantation mouse embryos, leading to embryonic malformations (Little & Mirkes, 2002). Different pathologies resulting from deregulation of cell death will be discussed in more detail later.

Diagram 1. The complex signaling pathways in cell death.



2. Importance of cell death in homeostasis

In vertebrates, the developmental processes of morphogenesis, remodeling and regeneration are sustained at high levels of many tissues throughout their life span. Cell

death has been illustrated to play an equally important role in an organism's homeostasis as it does in development (Pollack et al, 2002).

The homeostasis of different tissues relies on the balance between cell death and proliferation (Pollack et al, 2002). In the strictly regulated dynamic changes of lymphocytes, cytokine IL-15 is an essential mediator for the peripheral NK (natural killer) cell homeostasis, regulating the equilibrium between cell death and survival via maintenance of the anti-apoptotic bcl-2 (Ranson et al, 2003). In the rat small intestine, the epithelial homeostasis is balanced by the regulation of cell death and proliferation. In rats with ventromedial hypothalamus lesions, decreased intestinal mucosal apoptosis induces hyperphagia and obesity (Fujimoto et al, 2002). PCD is essential for the homeostasis of the immune system by removal of activated and self-reactive lymphocytes in the periphery. For example, cytotoxic T lymphocytes induce apoptosis in infected cells. After the immune response, the activated T cells in the periphery are removed by apoptosis, thus maintaining homeostasis (Kagi et al, 1994; Dennert, 2002).

Cell death also plays an important role in the turnover of different cells and serves as an adaptive mechanism to ablate neoplastic cells (Bredesen, 1995; Arase & Lanier, 2002). Hair cells in the vestibular organs of birds have a relatively short life span and die spontaneously to be quickly replaced by new hair cells. Studies have found that enhanced numbers of macrophages are recruited to sites of dying hair cells, suggesting that macrophages and their secretory cytokines-mediated cell death may be involved in hair cell regeneration (Warchol et al, 2001). Cell death plays a key role in the physiological control of cutaneous homeostasis. Non-neuronal nicotinic acetylcholine receptors (nAChRs) are abundantly expressed in skin, among which alpha7 nAChR is

found to stimulate keratinocyte apoptosis. Elimination of the alpha7 nAChR decreases relative amounts of the pro-apoptotic Bad and Bax at both the mRNA and the protein levels, leading to decreased amounts of the extracellular matrix proteins collagen 1alpha1, elastin and the metalloproteinase-1 in the skin of alpha7 knockout mice (Arredondo et al, 2003). Gastrointestinal epithelial cells also have a quick cell turnover, in which apoptosis plays a major role and the attenuation of apoptosis leads to neoplastic transformation (Obst et al, 2002). Upon DNA-damaging stimulation, activation of the tumor suppressor protein p53 may trigger demise of the cell in order to stop proliferation of cells with abnormal chromosomes (Chernova et al, 1995; Artandi, 2002; Su et al, 2002). Correspondingly, transgenic mice overexpressing the anti-apoptotic bcl-2 in lymphocytes are more susceptible to tumorigenesis than wild-type littermates (Strasser et al, 1990; Marsden et al, 2002).

Therefore, cell death plays an indispensable role in an organism's homeostasis. Increasing studies have indicated that abnormal cell death is always involved in a variety of pathologies.

3. Importance of cell death in pathogenesis

As well regulated cell death is crucial for development and maintenance of homeostasis, abnormally induced cell death will lead to abnormalities in development and homeostasis, including cancer, stroke, autoimmune and neurodegenerative diseases. Loss of the ability to die or inadequate cell death is a prominent characteristic of cancerous cells (Hager & Hanahan, 1999; Hofmann et al, 2002). The resistance of some tumor cells to induction of cell death by irradiation and chemical compounds is a big

challenge to cancer therapy (Kondo et al, 1995; Sax et al, 2002). Tumorigenesis is not the only consequence of inappropriate survival of cells. Immune systems are prone to damage resulting from abnormally prolonged survival of activated lymphocytes. For example, the long-lived B lymphocytes in bcl-2-overexpressing transgenic mice or bim-deficient mice result in prolonged humoral immune responses and pathological accumulation of plasma cells, which can eventually lead to fatal systemic lupus erythematosus-like autoimmune disease (Strasser et al, 1991). Moreover, too little cell death can also seriously damage health. For instance, there are mutations in the gene encoding perforin (a cytotoxic granule constituent required for apoptotic killing of target cells) in familial hemophagocytic lymphohistiocytosis (FHL) patients, suggesting a lack of apoptosis triggering within the immune system leading to a non-malignant accumulation of activated T lymphocytes and histiocytes in the reticuloendothelial systems, which is the major characteristics of FHL (Fadeel et al, 2001).

Too much cell death can break the balance between cell death and cell survival as well. Human immunodeficiency virus (HIV) infection results in an elevation of both CD4+ and CD8+ T cell death due to T cell activation, leading to impairment of the immune defense system (Galati et al, 2002). In the adult nervous system, inappropriately high levels of cell death lead to the pathogenesis of a number of neurodegenerative diseases characterized by loss of neurons, including Alzheimer's, Parkinson's, and amyotrophic lateral sclerosis (ALS) (for review, see Jellinger, 2001), as well as neuronal injuries (Yakovlev et al, 1997).

Although physiological and pathological cell death share similar molecular mechanisms in the execution phase, there are key differences in the way cell death is

triggered. In physiological cell death of the developing brain, trophic factor withdrawal plays a prominent role in initiation of cell death. Genetic mutations or environmental factors often stimulate pathological cell death in the adult brain, leading to neurodegeneration. In experimental animal models of ischemic brain injury, a large number of cells die after ischemia and during reperfusion (Li et al, 1995; Martin et al, 1998). The neurotoxicity of amyloid-β (Aβ) is pivotal in pathology of Alzheimer's disease. Aβ might induce apoptosis in neurons by interacting with different neuronal receptors, including 1) the receptor for advanced glycation endproducts (RAGE), which can mediate free radical production (Yan et al, 1996), 2) the p75 neurotrophin receptor, which can induce neuronal cell death (Yaar et al, 1997), 3) and the amyloid precursor protein, which can also induce neuronal cell death (Lorenzo et al, 2000). ALS is characterized by the degeneration of motor neurons in the spinal cord and brain, leading to paralysis. This degeneration appears to be related to free radical production. Mutations of the gene encoding superoxide dismutase (SOD-1) have been widely found in familial ALS (Rosen et al, 1993). Transgenic mice over-expressing mutant forms of SOD-1 show progressive motor neuron degeneration, whereas mice deficient in or over-expressing wild type SOD-1 do not develop motor neuron pathology (Cleveland, 1999). The pro-apoptotic activity of mutant SOD-1 in cultured neural cells suggests a role of apoptosis in ALS (Rabizadeh et al, 1995). DNA strand breaks have been found in brain of patients with Alzheimer's (Anderson et al, 1996) or Huntington's disease (Portera-Cailliau et al, 1995), and are also associated with oxidative damage that is often linked with increased neuronal vulnerability to death (Stadelmann et al, 1998).

The deregulated cell death in pathology further elucidates the importance of a tightly controlled cell death in an organism's healthy living. In attempt to gain a control of cell death both temporally and spatially, many signaling pathways need to be well regulated and coordinated, since cell death is actually regulated by many pathways. Some of these pathways will be discussed in more detail in the following chapter.

Chapter II. Mechanisms of Cell Death

1. Types of Cell Death
   A. Apoptosis
   B. Autophagy
   C. Necrosis
   D. Other
2. Pathways Involved in Cell Death
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      b. Expression and activity of Cdk5 in cell death
   C. Regulation of Cdk5 in Cellular Function
      a. Regulation of Cdk5 in differentiation
      b. Regulation of Cdk5 in cell death

As discussed in the previous chapter, cell death, as important as cell proliferation, plays an essential role in both development and homeostasis. The fact that many different types of cells can undergo cell death under various conditions suggests that there may be different types of cell death, and different cell death signaling pathways involved in this tightly regulated process.

1. Types of cell death

Many types of cell death have been observed under different conditions by many researchers. Originally, there were enormous disputes over the distinction of different types of cell death. To date, by both morphological and biochemical criteria, three major forms of cell death have been identified: apoptosis, autophagy, and necrosis. Apoptosis and autophagy are sometimes termed type I programmed cell death and type II programmed cell death, respectively. The term "programmed cell death" (PCD) was first introduced by RA Lockshin and CM Williams (Lockshin and Williams, 1964) to describe a type of predictable cell death during development, which programs cells to die following a developmental plan of the organism. Today, PCD specifically refers to a cell's built-in genetic program composed of many signaling pathways leading to the destruction of the cell.

A. Apoptosis

Apoptosis originally referred to the morphological features of cell death during development and tissue homeostasis (Kerr et al, 1972). Apoptotic cell death is characterized by a series of morphological changes, including cell shrinkage, membrane

blebbing, nuclear condensation, chromatin segregation, formation of membrane-bound apoptotic bodies, and internucleosomal DNA cleavage (Saraste and Pulkki, 2000). During apoptosis, the dying cell rounds up and condenses with volume shrinkage, but still maintains its membrane integrity. The chromatin coalesces into one or a few masses along the nuclear membrane, a process described as nuclear margination. Meanwhile, the cytoplasm fragments and plasma membrane forms blebs, which results in the formation of membrane-bound apoptotic bodies of the whole cell. The engulfment of the apoptotic bodies is accomplished by either professional phagocytes or phagocyte-like cells derived from neighboring cells through a process called phagocytosis (Lang et al, 2002; Shetty et al, 2002; for review, see Zakeri & Lockshin, 2002). The morphological changes of apoptosis result from a series of genetically programmed biochemical changes initiated in apoptotic cells. Mitochondria play a key role in the commitment of cells to apoptosis. During some forms of apoptosis, mitochondria remain intact and appear normal to shrunken rather than swollen, but mitochondrial membranes have lost polarity and permitted the release of cytochrome c and some other components, such as AIF (apoptosis inducing factors) and Diablo/Smac (an inhibitor of IAPs-inhibitor of apoptosis proteins), from the intermembrane space to cytosol (Liu et al, 1996; Madesh et al, 2002). The release of these intermembrane contents results in activation of caspases, which will further lead to activation of caspase-activated DNase (CAD) by inactivating the endogenous CAD inhibitor (ICAD). Activated CAD can cleave DNA between nucleosomes, giving rise to DNA fragments that are identified as a ladder on DNA electrophoresis gel (McIlroy et al, 1999; Kondratyev et al, 2002; Nagata et al, 2002). Caspases activation is also a hallmark of apoptosis. Although cell death can occur in the

complete absence of caspases, inhibition of caspase activity has been shown to abolish apoptotic changes during cell death (Leist & Jaattela, 2001; Glassford et al, 2002). The phagocytosis is mediated by the appearance of phosphotidylserine on the external face of the cell membrane from the internal face, resulting in the loss of cell membrane asymmetry in an apoptotic cell (Savill, 1997; 1998; Fadok et al, 1998; Huang et al, 2002). Since there is no release of proinflammatory cellular contents to the surrounding environment before the engulfment of apoptotic cells, apoptosis is immunologically "silent" (Fadok et al, 1998; Ronchetti et al, 1999). Apoptosis is an energy-requiring process and energy is required to achieve the specific apoptotic morphology and to translocate phosphotidylserine (Schlegel et al, 2000). During embryonic development, apoptosis is the predominant cell death. However, in situations where energy is compromised, cells may begin to die by an apoptotic mechanism but fail to complete the whole process, and continue to die via a different mechanism (for review, see Zakeri & Lockshin, 2002).

B. Autophagy

For the elimination of cells having massive cytoplasm, autophagic cell death or type II PCD is activated. These cells are usually large, quiescent or postmitotic cells, including muscle, differentiated neurons, and glandular tissues, such as insect glands at metamorphosis and mammary epithelium (Bowen et al, 1996; Lee et al, 2002). In this type of cell death, although many apoptotic changes eventually develop, they appear at a much later stage after the dying cell has undergone substantial alterations. It has been evaluated that classic apoptotic stage accounts for only about 10% of the whole period of

autophagy in which the dying cell is identifiable, and most of the tests for classic apoptotic characteristics to identify autophagy will produce negative results (Jochova et al, 1997). In cells undergoing autophagy, degradation of the cytoplasmic contents occurs before nuclear collapse, and intracellular membranes rearrange. Due to the bulky cytoplasm, large cytosolic vacuoles, derivatives of lysosomes, develop to digest cytoplasmic components within the cell (Thummel, 2001). The formation of large autophagic vacuoles makes the most prominent characteristic of autophagy. In autophagy, the dying cell maintains enough ATP levels for the completion of autodigestion of cytoplasm with a decreased number of mitochondria in cytoplasm that are still intact (Thummel, 2001). Caspase activity is not required to exhibit autophagic changes. In caspase-3 deficient MCF-7 cells, cell death induced by anti-estrogens tamoxifen and ICI 164 384 is predominantly, if not exclusively, autophagy (Bursch et al, 1996). When about 80% of the cytoplasm has been destroyed, some apoptotic changes will become detectable, such as condensation of remaining cytoplasm and fragmentation and margination of chromatin. At this stage, DNA electrophoresis reveals a classic DNA ladder (Zakeri et al, 1993; Dai & Gilbert, 1999). In some cases, cell death can be completed with phagocytosis (Schweichel & Merker, 1973; Clarke, 1990; Jochova et al, 1997; Thummel, 2001). Like apoptosis, autophagy is "immune silent" and does not provoke inflammation (Thummel, 2001).

However, apoptosis and autophagy may occur simultaneously within the same dying cell, which will share the features of both types of cell death. This "mixed" type of cell death has been found in sympathetic neuronal cell death (Xue et al, 1999), in antiestrogen-induced mammary carcinoma cell death (Bursch et al, 1996), and during

death of liver cells treated with etoposide (Qi & Sit, 1998).

C. Necrosis

In contrast to physiological cell death, necrosis is always described as an uncontrolled cell death. Necrosis occurs when sudden insults or severe and acute injuries, such as abrupt anoxia, deprivation of nutrients, and irradiation, occur in both physiological and pathological situations (Chautan et al, 1999; for review, see Denecker et al, 2001). Necrotic cells always have compromised ATP-generating system and membrane integrity, which leads to the swelling and bursting of organelles and eventually the cell. The release of bulk of cytoplasmic contents results in the classic consequence of necrotic cell death: inflammation. There is evidence for regulated necrosis under the control of a program different from apoptosis (Kitanaka & Kuchino, 1999), in contrast to the idea that necrosis is not a regulated process. However, necrosis still has its very unique characteristics distinct from apoptosis and autophagy: cellular swelling, loss of the integrity of plasma membrane, swelling and bursting of organelles, large DNA degradation. In some cases, necrosis can arise from apoptosis. For instance, in cases of acute liver toxicity, cells most exposed to the toxin (closest to the central vein) undergo necrosis, and the more peripheral cells may die by apoptosis or start with apoptosis but later convert to necrosis (Sun et al, 2000).

D. Other

Apoptosis, autophagy and necrosis are three major types of cell death, but sometimes a cell might die in a way that does not fit into any of these categories. Unlike

classical apoptosis, there is "apoptosis-like" PCD, in which the chromatin condenses to lumpy masses that are less compact than in apoptosis and phagocytosis recognition molecules, or phosphotidylserines, are displayed on the outer plasma membrane before the cell lyses. Any degree and combination of other apoptotic features can be found in this type of cell death. Additionally, apoptosis-like PCD can be dependent or independent of caspases (for review, see Leist & Jaattela, 2001; Mathiase & Jaattela, 2002). For instance, mouse embryonic stem cells microinjected with apoptosis-inducing factor (AIF) are triggered to undergo apoptosis-like PCD with lumpy chromatin condensation (Susin et al, 1999). In caspase-3 deficient MCF-7 cells, depletion of Hsp70 activates an apoptosis-like PCD exhibiting an incomplete chromatin condensation (Nylandsted et al, 2000). Many other caspase-independent cell deaths can be addressed by this description. For example, in methionine-dependent carcinoma PC13 cells, methionine restriction induces caspase-independent cell death with bid cleavage and bcl-2 reduction (Lu et al, 2003). In addition, the interruption of active cellular process of classical apoptosis can lead to "necrosis-like" PCD. This type of cell death is characterized by absence of chromatin condensation or, at best, with chromatin clustering to loose speckles. A varying degree of apoptosis-like changes including phosphotidylserine exposure might occur prior to cell lysis. Aborted apoptosis usually falls into this category (for review, see Mathiase & Jaattela, 2002). Cells die through necrosis-like PCD upon treatment with oxygen-radical scavengers (Vercammen et al, 1998), inhibition of poly(ADP)-ribose polymerase (PARP, a DNA damage repair enzyme) (Ha et al, 1999), or mutations in intracellular signaling molecules (Holler et al, 2000).

Notably, cell death that comprises both apoptosis and necrosis might occur during cell death progression. For an instance, during brain ischemia (Beilharz et al, 1995) or liver damage induced by death domain receptor ligands (Leist et al, 1995; 1996), injured cells undergo both apoptosis and necrosis. In dying neurons, if the intracellular energy is deprived at early stages, cell death is going to take the appearance of necrosis (Ankarcrona et al, 1995) or a mixture of necrosis and apoptosis (Volbracht et al, 1999).

A novel form of PCD, oxytosis, was recently identified. Oxytosis is a unique oxidative stress-induced PCD pathway, in which there is a sequential requirement for de novo macromolecular synthesis, lipoxygenase activation, reactive oxygen species production, and the opening of cGMP-gated channels, to allow the influx of extracellular calcium. Besides apoptosis, oxytosis is another type of neuronal cell death during the development of the CNS as well as in trauma and neurodegenerative diseases. Oxidative glutamate toxicity is the most widely studied type of oxytosis, in which exogenous glutamate inhibits cystine uptake through the cystine/glutamate antiporter leading to a depletion of glutathione. It has been found that the translation initiation factor elF2alpha plays a central role in this pathway by regulating the levels of glutathione (Tan et al, 2001).

Given the various types of cell death, identifying different types of cell death at a general biological level using unique biomarkers is crucial to understand the mechanisms of cell death. The mode of cell death might have differential effects on the surrounding tissue (Savill & Fadok, 2000), and might control the horizontal spread of oncogenic inflammation and infection (Bergsmedh et al, 2001; Boise & Collins, 2001). In this thesis, we will in most cases evaluate the regulation of Cdk5 activation during apoptotic

cell death, and start to ask the question whether this regulation is the same in other modes of cell death.

2. Pathways involved in cell death

Cell death occurs through multiple independent pathways that are initiated either within the cell (intrinsic) or from outside the cell (extrinsic). Most cell death signaling pathways converge on a common cell destruction machinery, which is the activation of a family of cysteine proteases (caspases). The removal of doomed cells is accomplished by proteolysis of cellular components, DNA degradation, phagocytosis by neighboring cells, or autophagy via activation of the cell's own lysosomes.

A. P53 pathway

a. Identification of p53

The p53 protein was first identified as a cellular protein immunoprecipitated with the SV40 large T antigen, a product of the SV40 viral genome (Lane & Crawford, 1979; Linzer & Levine, 1979). The tumor suppressor function of p53 was identified when several groups demonstrated that cells transfected with wild type p53 cDNA exhibited growth arrest or apoptosis (Finlay et al, 1989; Baker et al, 1990; Yonish-Rouach et al, 1991). The importance of p53 gene in suppressing tumor development has been illustrated by the fact that p53 is disrupted in nearly 50% of all human tumors (Serrano et al., 1997). Although mouse embryos deficient for p53 develop normally suggesting that p53 is dispensable for normal progression of the cell cycle, p53 null mice are susceptible to lymphomagenesis, which results in an average lifespan of only 5 months (Donehower

et al, 1992).

b. Regulation of p53

Levels of p53 are normally low owing to the short half-life of p53 protein. However, the stabilization and activation of p53 can be induced by different mechanisms. Both intracellular and extracellular stress signal can evoke p53 stabilization and activation. For example, DNA-damaging agents, such as ultraviolet or γ irradiation and chemotherapeutic drugs, can stabilize and activate p53 by covalent modification (for review, see Bulavin et al, 2002). Phosphorylation of the transactivation domain on p53 gene is always involved in this modification. The ATM (ataxia telangiectasia mutated) and ATR (ATM-related) kinases phosphorylate p53 after ionizing radiation and ultraviolet irradiation, respectively (Banin et al, 1998). Consequently, cells that lack expression of either of these genes or their other known substrates, CHK1 and CHK2 (which also phosphorylate p53), display reduced stabilization and activation of p53 in response to DNA-damaging agents (Chehab et al, 2000). P53 may also be stabilized and activated by internal aberrations such as the deregulation of cellular oncogenes. Overexpression of some oncogenes induces expression of $p14/19^{ARF}$, which by inactivating MDM2 (Mouse Double Minute 2, an endogenous negative regulator of p53) blocks p53 degeneration (for review, see Michael & Oren, 2002). Other oncogenes otherwise can inhibit p53. For example, unlike its homologues ASPP1 and ASPP2 in ASPP (Apoptosis Stimulating Proteins of P53) family, the newly identified iASPP oncoprotein is a key inhibitor of p53 conserved from worm to human (Bergamaschi et al, 2003). Once activated, p53 can induce either cell cycle arrest or apoptosis. After DNA damage, p53 expression blocks progression of the cell cycle until the DNA can be

repaired; alternatively, if the damage is extensive and severe, the cells enter apoptosis (for review, see Vousden & Lu, 2002).

c. p53-mediated cell death

P53, as a transcription factor mediating both transactivation and transrepression of target genes, plays a crucial role in the execution of apoptosis (Lowe et al., 1993; Clarke et al., 1993; for review, see Hickman et al, 2002). Accordingly, p53 mutants by point mutation in the transcriptional activation domain effectively cripple the apoptotic activity of p53 (Chao et al, 2000). Neurons from p53-null mice are more resistant to excitotoxins, DNA-damaging reagents, or hypoxia compared to their wild type counterparts (Xiang et al, 1998; Halterman et al, 1999; Morris et al, 2001). Activated p53 regulates the expression of several apoptotic mediators including members of the bcl-2 and TNF (tumor necrosis factor) receptor families. Therefore, p53-mediated or –dependent cell death can take either mitochondria-dependent or death receptor-dependent pathway.

The principal role of p53 during cell death is through the mitochondria-mediated pathway. Among p53-mediated genes, bax was the first apoptotic factor to be identified as a target of p53 transactivation (Miyashita et al, 1994). Several lines of evidence suggest that p53 could trigger cell death machinery through induction of the release of cytochrome c from mitochondria mediated by bax translocation. In L929 fibroblasts treated with etoposide, the cell death cascade proceeds by activation of p53 through phosphorylation, p53-dependent up-regulation of bax, translocation of bax to mitochondria, and release of cytochrome c (Karpinich et al, 2002). Using ventricular myocytes with overexpression of p53, a significant elevation of bax and MDM2 gene is observed, and the mitochondrial death pathway is activated by p53 activation (Regula &

Kirshenbaum, 2001). In human CD4+T cells infected with HIV-1, p53 phosphorylation is followed by increased mRNA and protein expression of p21, bax, HDM2 (the human homologue of MDM2), cytochrome c and AIF release from mitochondria, activation of caspases, and up-regulation of surface FasL (Fas ligand) expression, in sequence, indicating the involvement of a p53-mediated mitochondrial pathway in HIV-1 induced cell death (Genini et al, 2001). P53 also represses the expression of anti-apoptotic bcl-2 and thereby contributes to apoptosis by blocking survival signals mediated by bcl-2 (Miyashita et al, 1994; Lyer et al, 2003). In cell death mediated by p53 in response to oxidative stress, p85, a regulator of phosphatidyl-3-OH kinase, plays an indispensable role (Yin et al., 1998). During apoptosis in human lymphoblastoid cells exposed to nitric oxide, the increase of Apaf-1 expression and reduction of XIAP (X-linked inhibitor of apoptosis) protein level are p53-dependent (Li et al, 2002). In p53 null Saos-2 cells, expression of wild-type p53 induces cell death, accompanied by cytochrome c release and caspase activation (Schuler et al, 2000).

P53 also mediates cell death through the death receptor pathway. The death receptor ligand Fas/apo1 can be induced by p53, indicating that p53 may induce caspase activation through death receptor signaling (Owen-Schaub et al, 1995; Gadducci et al, 2002). In human cancer cell lines, overexpression of p53 results in apoptosis with activation of caspases, including caspase-8 (Liu et al, 2003). In mice with 5 Gy ionizing radiation, there is a p53-dependent induction of death receptors KILLER/DR5, FAS/APO1, and EI24/PIG8 in different tissues, which is not detectable in p53 null animals, suggesting the involvement of death receptors in p53-mediated cell death (Burns et al, 2001). In the human colon cancer cell line DLD-1, p53 activation induces cell

death with upregulated levels of death receptors, Fas, TRAIL-R1 and TRAIL-R2 (Bartke et al, 2001).

Recently, some novel p53-regulated genes have been identified. Among them are genes encoding two BH3 (Bcl-2 homology 3) domain proteins, PUMA (Nakano et al, 2001) and NOXA (Oda et al, 2000), which associate with anti-apoptotic bcl-2 family proteins and promote release of cytochrome c. Other genes include NF-κB, a mediator of TNF receptor signaling (Ryan et al, 2000); p53AIP, which dissipates mitochondrial potential (Oda et al, 2000); and PIDD, a novel death domain protein that participates in death receptor signaling (Lin et al, 2000). Moreover, Apaf-1, which encodes an adaptor protein that enables the cytochrome-c-dependent activation of procaspase-9, has been identified as a direct transcriptional target of p53 (Moroni et al, 2001).

d. p53-independent cell death

P53-independent cell death has also been addressed, although its mechanism remains poorly understood. During G1-S transition, inhibition of Cdks (2, 4 and 6) can induce cells to undergo apoptosis in a p53-independent manner (Shibata et al, 1996). P19(ARF) is a potent tumor suppressor. By inactivating MDM2, p19(ARF) upregulates p53 activity to sensitize cells to apoptosis in response to stumuli. However, the expression of p19(ARF) can induce apoptosis in primary mouse embryonic fibroblast lacking p53/ARF, indicating a p53-independent pathway for p19(ARF)-induced apoptosis (Tsuji et al, 2002). Retinoid-induced apoptosis in B-cell chronic lymphocytic leukemia cells is independent of p53, but requires caspase-3 activation (Pepper et al, 2002). Dlk/ZIP kinase is one of five members of the death associated protein (DAP) kinase family. DAP kinase is able to induce apoptosis in a

p53-dependent manner. Notably, Dlk/ZIP kinase has been demonstrated to trigger mitochondrial apoptosis pathway in p53-deficient mouse fibroblast cells (Kogel et al, 2003). Therefore, the above evidence suggests that p53-independent cell death also plays an important part in the demise of various cells.

Taken together, p53-dependent and –independent cell death are two key signaling pathways involved in the cell death machinery. In this thesis, we will therefore explore the regulation and activation of Cdk5 in both p53-dependent and –independent cell death, in order to find out how Cdk5 activation fits with the major known cell death signaling pathways during cell death.

e. Other members of p53 family

The p53 tumor suppressor protein belongs to a small family of structurally and functionally related proteins, which includes two other members, p63 and p73. All three proteins share sequence similarity, although p63 and p73 are more similar to each other than to p53 (for review, see Yang et al, 2002). P73 is highly expressed in airway epithelia, and newborn p73-/- mice display airway inflammatory responses in the absence of provocative bacterial infections (for review, see Yang et al, 2002). Studies have illustrated that p73 functions in many situations, such as cell cycle arrest (De Laurenzi et al, 1998), apoptosis (Jost et al, 1997), neuronal differentiation (De Laurenzi et al, 2000a), and epidermal differentiation (De Laurenzi et al, 2000b). P73 regulates the expression of many genes, some of which are also p53-target promoters (De Laurenzi et al, 1998). It is becoming evident that p73 has activities that are not shared by p53. p73 has been shown to activate promoters that do not contain consensus p53-binding sites (Takagi et al, 2001), indicating that p73 has a specific role in normal development. P73 is able to drive

the transcription of several apoptotic effectors that are also induced by p53, including bax, p53AIP1 (Constanco et al, 2002), and NOVA (Flores et al, 2002). In addition, p73 might contribute indirectly to the activation of apoptosis by modulating p53 function. The physical binding of p73 to MDM2 might sequester MDM2 and lead to an increase in the steady-state level of p53 and its apoptotic function (for review, see Melino et al, 2002).

Unlike the conditionally expressed p53, p63 is constitutively present, especially in the stem cell compartment of many epithelial tissues (Yang et al, 1998). P63-null mice are born but lack limbs and a wide range of epithelial structures including skin, prostate, breast and urethra (Yang et al, 1999). These findings suggest that p63 function is involved in epithelial cell maintenance and essential for development of skin, limbs and appendages, breast, urothelium and prostate (for review, see Yang et al, 2002).

B. Caspase cascade

Although different signaling pathways exist in response to different stimuli, they usually converge to a common mechanism leading to cell demise. Cysteinyl aspartate-specific proteases, the so called "caspases", are the main enzymes involved in this caspase cascade. To date, there are at least 14 mammalian caspases identified (Weil et al, 1996; Bilsland & Harper, 2002). Caspases are constitutively expressed as zymogens with very low enzymatic activity, and they are homologues to the most studied death effector Ced-3 in *C. elegans* (Yuan et al, 1993). Upon activation by either self-processing or other caspase-mediated cleavage, the fully active caspases are a heterotetramer composed of two identical subunits of ~20 kDa and two identical subunits of ~10 kDa (Nunez et al,

1998). Based on the tetrapeptide recognition sequence in the substrate, caspases can be categorized into three groups, WEHDases (caspase-1, -4, and –5), DExDases (caspase-2, -3, and –7) also termed as effector/executioner caspases, and (IVL)ExDases (caspase-6, -8, -9 and –10) referred to initiator/activator caspases (Nicholson & Thornberry, 1997).

Different members of the caspase family cooperate to direct cells to their demise. There is abundant evidence suggesting that the combination of different caspases is part of a common core death process in apoptosis (Henkart, 1996; Bilsland & Harper, 2002; Lu et al, 2003). So far, there are two main cell death pathways involving the activation of caspases. One pathway is extrinsic, initiated by the association of death factor to its ligand, leading to activation of the initiator caspase 8 by death receptor recruitment, followed by activation of downstream effector caspases, such as caspase 3, -6, and –7 (Juo et al, 1998; Rokhlin et al, 1998). The second pathway is intrinsic, triggered by the release of cytochrome c from the intermembrane space of mitochondria upon mitochondrial permeablization transition, leading to the formation of apoptosome, a complex of Apaf-1/cytochrome c. The proteosome recruits and activates pro-caspase-9. The activated caspase-9 cleaves and activates the downstream effector caspase-3, -6 or –7 (Slee et al, 1999; Jiang et al, 2003). Activated caspases digest many cellular proteins, including proteins responsible for cell cycle regulation (e.g. RB, MDM2, Los et al, 2001), DNA damage recognition and repair (e.g. DNA-PK, p53, PARP, Martin & Green, 1995), and regulation of the cellular structure (e.g. actin and lamins) (Moretti et al, 2002). The consequence of the digestion of these functional or structural proteins has been linked with the typical apoptotic morphology, i.e. DNA fragmentation that gives rise to the classic DNA ladder on agarose gel, during both developmental and pathological demise

(Sun et al, 1999; for review, see Leist & Jaattela, 2001). Correspondingly, Fas-induced cell death is not always apoptotic unless caspase 3-like proteases are functional (Toyoshima et al, 1997).

As mentioned previously in the Introduction, studies on caspase knockout mouse models have shown caspases play a pivotal role during development. The activation of different caspases is also involved in pathogenesis of either neuron injury or degeneration. For instance, in rat brain after a transient global ischemia, caspase-3 is activated at least partially through a caspase-9-dependent activation, since use of specific caspase-9 inhibitor before ischemia attenuates caspase-3 activity and significantly enhances neuronal survival (Cao et al, 2002). Caspases 3, 8, and 9 are activated leading to the inappropriate cell death in neurodegenerative diseases, including Alzheimer's, Parkinson's, and Down syndrome. And inhibiting caspase activity by members of the IAP (inhibitor of apoptosis protein) family results in neuroprotection in Aβ-induced neuronal cell death (for review, see Troy & Salvesen, 2002). Intracerebroventricular injection of vinscristine, a microtubule-depolymerizing agent, causes massive apoptosis of cerebellar granule cells accompanied with caspase-3-like protease activation, leading to motor dysfunction (Shimizu et al, 2002). Deregulation of caspases also plays roles in other pathology, such as cancers. Failure to activate caspases, such as caspase-3, has been linked with the resistance of cancer cells to cell death induction during treatment (Choi et al, 2002).

Because of the catastrophic effects of caspases on a cell's fate, it is of extreme importance for the activation of caspase cascade to be highly regulated. There has been impressive progression in elucidating key regulatory elements of caspase-mediated cell

death pathways. For example, bcl-2 family members regulate cytochrome c release from mitochondria (Vander Heiden & Thompson, 1999; Cory & Adams, 2002; Kuwana et al, 2002). Bid or its BH3-domain peptide can activate Bax to produce supramolecular opening on the outer mitochondrial membrane, allowing the passage of large molecules, such as cytochrome c, during apoptosis. This process is inhibited by the anti-apoptotic Bcl-xL (Kuwana et al, 2002). Inhibitors of apoptosis proteins, or IAPs, such as XIAP, ML-IAP, cIAP-1 and cIAP-2, are direct inhibitors of caspases, and are themselves negatively regulated by the mitochondrial proteins Smac/Diablo and Omi/HtrA2, which are coreleased with cytochrome c (Ekert et al, 2001; Verhagen & Vaux, 2002). Additionally, IAPs may also play a role in mediating the removal of caspases through the ubiquitin-proteosome degradation pathway (Huang et al, 2000). Very recently, Wang's group has identified the oncoprotein prothymosin-α (ProT) and the tumor suppressor putative HLA-DR-associated proteins (HDAPs) as important regulators of caspase activation. Both proteins appear to mediate distinct steps in the mitochondrial cell death pathway: ProT blocks formation of the apoptosome, which is supported by the finding that RNA interference directed against ProT activity sensitizes cells to apoptosis; in contrast, HDAP appears to facilitate apoptosome-mediated caspase-9 activation (Jiang et al, 2003).

In summary, the activation of caspases plays an essential role in cell death and an orchestrated regulation of their activation is required for the execution of the doomed cells. In view of the essential role that caspases play during cell death, in this thesis, we will evaluate the dependency of Cdk5 activation on the activity of different caspases (3, 9 and Apaf-1) during cell death.

C. Non-caspase proteases

Although the inhibition of caspase activation often delays cell death, it does not necessarily confer long-term survival. Instead, it might reveal, or in some cases even enhance, underlying caspase-independent death program, which can be divided into apoptosis-like PCD and necrosis-like PCD according to the nuclear morphology of dying cells, as discussed previously. Like classic apoptosis, caspase-independent PCD is often dependent on proteases and mitochondrial membrane permeabilization (MMP). The most extensive evidence linking non-caspase proteases with cell death originates from studies of calpains, cathepsins, and serine proteases (Sarin et al, 1995; Talanian et al, 1997; Pennacchio et al, 1998; Johnson, 2000; Leist & Jaatela, 2001). These proteases often work with caspases in classic apoptosis, and can also trigger cell death and bring about some morphological changes characteristic of apoptosis in a caspase-independent manner (Johnson, 2000; Leist & Jaatela, 2001).

a. Calpain

Calpains are cysteine proteases that reside in the cytosol in an inactive zymogen form (John, 2000). Two isoforms of calpains, m- and μ-calpains, are ubiquitously expressed in mammalian tissues and have been linked to cell death. Activation of calpains requires an elevation in intracellular calcium. Proteolytic cleavage and association with membrane phospholipids further contribute to calpain activation, possibly by lowering the calcium requirement. The active forms of calpains are comprised of a heterodimer of 78 kDa-18 kDa, resulting from cleavage of the inactive pro-enzyme heterodimer of 80 kDa-29 kDa (Wang 2000). Calpain activity is controlled

by calpastatin, an endogenous inhibitor of calpains that can be inactivated by calpain and caspase-mediated cleavage (for review, see Wang, 2000).

Originally, calpain activation was thought to be involved only in necrotic neuronal death in ischemic and excitotoxic neuronal injury (for review, see Yuen and Wang, 1998). Rapid and massive Ca^+ influx and subsequent calpain activation leads to general DNA degradation and dissolution characteristic of necrosis rather than condensation as observed in apoptosis. In rat developing brain, calpain activation is associated with excitotoxic/necrotic cell injury caused by hypoxia-ischemia (Han et al, 2002). Calpain activation in apoptosis was first demonstrated in thymocytes (Squier et al, 1994). To date, calpain activation has been linked to apoptosis in response to various stimuli, such as in TCR-treated mature lymphocytes (Sarin et al, 1995), staurosporine-insulted neuroblastoma SH-5Y5Y cells, NGF-deprived rat PC12 cells, and in low K^+ -stimulated rat cerebellar granule neurons (Nath et al, 1996a, 1996b). Calpains can participate in death signaling upstream or downstream of caspases (Waterhouse et al, 1998; Varghese et al, 2001). In post-mitotic neurons, wild type APP (amyloid precursor protein) increases the level of intracellular calcium, which activates calpain, followed by caspase-3 activation, resulting in cell death, indicating a calpain-mediated caspase-3 activation pathway in neurons (Kuwako et al, 2002). Furthermore, calpains can induce caspase-independent cell death. In transgenic mice expressing the baculovirus protein p35, a potent viral caspase inhibitor, expression of p35 fails to influence kainate-induced calpain activation and neuronal death in the same areas, indicating calpain might play a bigger role than caspases in kainate-induced neuronal excitotoxicity (Tomioka et al, 2002). Increasing attention is being paid to calpains as more evidence suggests an

involvement of activation of these proteases in neurodegeneration (Patrick et al, 1999; Lee et al, 2000).

b. Cathepsins

The cathepsin protease family is composed of cysteine, aspartate and serine proteases (Johnson, 2000; Turk et al, 2001). So far, cysteine cathepsins B and L and aspartate cathepsin D have been most clearly linked to PCD. Most cathepsins mature in the endosomal-lysosomal compartment. They can be activated by autoproteolysis in acidic pH or proteolysis by other proteases (e.g. cathepsin D can activate cathepsins B and L). The role of cathepsins in PCD has been provided by studies showing resistance against TNF-induced liver apoptosis in mice lacking cathepsin B (Guicciardi et al, 2000), and massive PCD in the brains of mice deficient for cysteine cathepsin inhibitor cystatin B (Pennacchio et al, 1998).

Cathepsins participate in both caspase-dependent and –independent PCD induced by a variety of stimuli, including death receptors, camptothecin, bile salt, oxidants and retinoids (Roberts et al, 1999; Guicciardi et al, 2000; Foghsgaard et al, 2001). In all of these models, cathepsins translocate from lysosomes to the cytosol and/or nucleus before the appearance of PCD morphological changes. Interestingly, the inhibition of cathepsins protects cells from PCD without preventing the release of cathepsins from lysosomes (Foghsgaard et al, 2001). Notably, in some cells, cathepsins might be pivotal for survival, as shown by cysteine cathepsin inhibitor CAT-1, which kills leukaemia and lymphoma cells (Zhu & Uckun, 2000).

c. Serine proteases

Serine proteases granzyme A and B are among the most prominent components of cytotoxic granules of cytotoxic T lymphocytes (Johnson, 2000). Studies employing mice lacking either granzyme A or B have demonstrated that granzyme B is required for the granule-induced, rapid caspase-mediated apoptosis (Heusel et al, 1994; Johnson, 2000). Granzyme B after entering the target cells cleaves a variety of cellular substrates after aspartate residue to induce apoptosis, including several caspases, PARP, DNA-PK, NuMA (Andrade et al, 1998), as well as the apoptotic nuclease complex CAD/ICAD (Thomas et al, 2000; Sharif-Askari et al, 2001). However, in the presence of caspase inhibitors, granzyme B triggers a slower necrosis-like PCD (Talanian et al, 1997). It has been long known that bid processing, cytochrome c release, and mitochondrial depolarization are associated with granzyme B-mediated cell death in different cells (Heibein et al, 1999; Sutton et al, 2000; Pinkoski et al, 2001). Thomas et al demonstrated that bid cleavage and cytochrome c release are not required for granzyme B to induce cell death in target cells (Thomas et al, 2001). Granzyme A is a trypsin-like protease that cleaves substrates after lysine or arginine residues. Death induced by granzyme A is associated with DNA single-strand breaks generated by a granzyme A-activated DNase (Beresford et al, 2001).

Omi/HtRA2, a newly identified serine protease, is also involved in cell death. Omi was originally identified via affinity chromatography as an XIAP-binding protein (Martins et al, 2001). The Omi precursor (50 kDa) resides in mitochondria where it is processed through auto-catalytic removal of its N-terminal mitochondrial translocation sequence to generate the mature 36 kDa Omi. Mature Omi carries an amino-terminal IAP-binding motif similar to that found in Smac/diabolo, caspase-9, and the Drosophila

Reaper, Hid and Grim (Goyal et al, 2000; Srinivasula et al, 2001). Apoptotic stimuli lead to translocation of Omi to cytosol, where it binds IAPs to enhance caspase-dependent apoptosis. Omi is also involved in caspase-independent cell death. Sequence analysis has identified a trypsin-like serine protease domain in Omi, and the serine protease activity is able to cleave casein (Suzuki et al, 2001). The serine protease activity of Omi has been shown to attribute to Omi-mediated caspase-independent cell death. Mutants of Omi lacking both protease activity and IAP-binding ability cannot induce spontaneous cell death or enforce chemotherapy-induced cell death. But forms of Omi retaining only protease activity will enhance cell death in Apaf-1 or caspase-9 null cells. ZVAD-fmk, a broad spectrum caspase inhibitor, is unable to inhibit cell death induced by overexpression of Omi protease activity (Hegde et al, 2001). Therefore, Omi is able to induce caspase-independent cell death without its IAP-binding activity.

Another serine protease, apoptotic protease 24 (AP24) has been shown to mediate DNA fragmentation in TNF-, UV- and chemotherapy-induced PCD of some cancer cells (Wright et al, 1998).

Taken together, the above evidence that the activation of so many different non-caspase proteases is involved in caspase–independent cell death suggests their important role in cell death process.

2. Cell cycle and cell death

Cell cycle progression and cell death are two separate but highly related events during a cell's life span with a number of genes involved in one process also playing

roles in the other.

A. Deregulation of cell cycle in cell death

Cell cycle deregulation has long been linked to induction of cell death. This argument came from the finding that apoptosis may result from an improper activation of a family of protein kinases normally involved in the regulation of mitosis (Ucker, 1991; Rubin et al, 1992). Many agents that induce apoptosis have been shown also to disrupt the cell cycle progression (Kung et al, 1990; Sen and D'Incalci, 1992). In the *in vitro* culture of human umbilical vein endothelial cells (HUVEC), when the cells enter an irreversible G1 growth arrest status referred to as replicative senescence, the senescent HUVEC display a considerable increase in spontaneous apotosis, implying a link between the disruption of cell cycle progression and cell death induction (Wagner et al, 2001). In rat thyroid PC Cl 3 cells, over-expression of HMGA1 (the high mobility group protein A1) causes apoptosis in which caspase-3 is activated, in coincidence with a disrupted cell cycle: cells enter S-phase earlier and the G2/M transition is delayed (Fedele et al, 2001). The G0/G1 arrest of cell cycle caused by green tea polyphenols ultimately leads to cell death in human epidermoid carcinoma A431 cells (Ahmad et al, 2000). The tumor suppressor p19(ARF) can upregulate p53 activity by inactivating MDM2 to induce cell cycle arrest and sensitize cells to apoptosis (Tsuji et al, 2002). Regulation of cell cycle is always altered in cancers, and understanding of the relationship between deregulated cell cycle progression and cell death has been of significance in the treatment of different cancers, such as prostate (for review, see Kyprianou et al, 2000) and ovarian cancers (Baldwin et al, 2003).

Many genes that are key players for cell cycle regulation are also involved in cell death. The transcription factor E2F-1 induces cell cycle progression at the G1/S checkpoint, and deregulation of E2F-1 provokes cell death in many malignant cells. Very recently, overexpression of E2F-1 in cancer cells has been shown to induce the expression and auto-phosphorylation of PKR, the ds-RNA-dependent kinase, leading to phosphorylation of downstream eIF-2α, causing apoptosis (Vorburger et al, 2002). The tumor suppressor protein, retinoblastoma protein (Rb), is an important cell cycle regulator and involved in mitosis, differentiation as well as cell death. In neonatal ventricular myocytes, hypoxia-induced apoptosis is dependent on Rb inactivation upon phosphorylation, and expression of constitutively active Rb protects cardiomyocytes from death (Hauck et al, 2002). The transcription factor NF-κB promotes cell survival, and its inhibition has been found to induce the loss of mitochondrial transmembrane potential and caspase-independent, calpain-dependent apoptosis in retinoblastoma cells (Poulaki et al, 2002).

However, among the many cell cycle-related genes, the members of cyclin-dependent kinase family are considered the master regulators of cell cycle progression.

B. Master regulators of cell cycle: Cyclin-dependent kinases (Cdks)

Eukaryotic cell cycle is regulated by the periodic activation of a family of proline-directed serine/threonine kinases, cyclin-dependent kinases (Cdks). By now, 15 Cdks have been identified, most of which are structurally and functionally related. Most of the functioning Cdks are heterodimeric complexes consisting of a protein catalytic subunit, the Cdk, and a cyclin activating subunit (Gupta et al, 2002). Regulation of cell cycle by

Cdk activity is achieved through a tight control of Cdk activity by three mechanisms: 1) via association with its activating subunit cyclins, 2) via phosphorylation by CAKs (Cdk-activating kinases) to allow maximal activation, and 3) via binding to CKI (Cdk inhibitors) to be inhibited.

Most Cdk-cyclin complexes are important in the control of cell division and proliferation. For example, the kinase activities of both Cdc2 and Cdk2 are detected in proliferating cells and are essential for cells to progress through the key transition points of cell cycle (Elledge and Spottswod, 1991; Koffetal, 1991; Meyerson et al, 1992). In leukemia L1210 cells arrested in G1 and accumulated in G2, the protein expression levels of Cdc2/cyclin B, Cdk2/cyclin A, and Cdk2/cyclin E significantly decrease (Meijer et al, 1997). Differential expression of Cdks regulates cell cycle progression in growth vs. differentiation. For instance, in ML-1 human myeloblastic leukemia cells, Cdk2/cyclin E is expressed at relatively high levels in growth, while Cdk5/cylin D1 is maintained at high levels in differentiation (Li et al., 1997). Expression of Cdks is also developmental stage-dependent. During postnatal development of rat heart, Cdk1, Cdk2, Cdk4 and Cdk5 abruptly decline to almost undetectable levels after 10-20 days; in contrast, Cdk7 slightly increases and Cdk8 does not change significantly (Kim et al., 1998).

As mentioned previously, disturbance of the normal cell cycle progression can lead to cell death. The activation of various Cdks has been identified in apoptosis (Shi et al, 1994; Meikrantz et al, 1994). In cell death of Tat (*HIV-1* transactivator protein) expressing cells, Cdc2 and Cdk2 are activated (Li et al, 1995). In bcl2-/- human HeLa cells, the overexpression of wild type Cdc2, Cdk2, Cdk3, or cyclin A markedly elevates the incidence of apoptosis induced by staurosporine and TNFα, which otherwise fail to

induce cell death in these cells (Meikrantz et al., 1996). The induction of cyclin B and activation of related Cdks have been found to occur prior to the commitment of neurons to both dopamine- and peroxide-triggered apoptosis (Shirvan et al., 1998). The activation of Cdk1, Cdk2 and Cdk5 is involved in axotomy-induced death in retinal ganglion cells (Lefevre et al, 2002). Cdk2 and Cdk6 are activated in embryonic cortical neuronal death evoked by camptothecin, a DNA-damaging agent (Ghahremani et al, 2002). Correspondingly, inhibition of the function of some Cdks can protect cells from cell death. Overexpression of bcl-2 reduces the protein levels of Cdc2 and Cdk2, coinciding with a suppressed apoptosis induced by staurosporine in HeLa cell (Meikrantz et al, 1994). Also in human HeLa cells, dominant negative mutants of Cdc2, Cdk2 and Cdk3 each suppress apoptosis induced by both staurosporine and TNFα (Meikrantz et al., 1996). Kainate (KA), an ionotropic glutamate receptor agonist, induces apoptosis in the primary cultures of murine cerebellar granule cells. This KA-mediated neurotoxicity and apoptosis can be partially blocked by the Cdk inhibitor, roscovitine (Giardina & Beart, 2002).

On the other hand, perturbation of Cdk activity may also lead to cell death. Inhibition of Cdks by Cdk inhibitors, such as olomoucine or roscovitine, enhances cell death induced by E2F-1 overexpression in human gastric carcinoma cells (Atienza et al, 2000). Treatment of Jurkat and KG1 cells with Cdk inhibitors results in an increase of apoptotic cells (Vermeulen et al, 2002). Inhibition of Cdk activity by overexpression of p27Kip1, a Cdk inhibitor, induces cell cycle arrest and apoptosis in human renal carcinoma cells (Katner et al, 2002).

Therefore, Cdks function as master regulators of cell cycle progression, and the

deregulation of their activation can result in cell death. However, among the members of Cdk family, there is one unique Cdk, whose functions identified so far are not relevant to cell division control. This is Cdk5.

3. Regulation of Cdk5

A. What is Cdk5?

Cdk5 is a member of Cdk family. Cdk5 was originally identified from bovine brain due to its close sequence homology (~60%) to the prototypic Cdk, human Cdc2 (Lew et al, 1992). Structurally, Cdk5 is highly conserved evolutionarily. It shares 99% identity at protein level among vertebrates, such as rat (Hellmich et al, 1992), mouse (Ino et al, 1994), bovine (Lew et al, 1992), *Xenopus* (Gervasi & Szaro, 1995), and human (NCBI, protein bank). Fruit fly Cdk5 has 77% amino acid identity with its mammalian counterparts (Hellmich et al, 1994). Although Pho85, the Cdk5 homologue in budding yeast, has only 57% protein sequence homology, it is functionally closely related to vertebrate Cdk5 (Huang et al, 1999). For example, overexpression of Cdk5 in yeast cells can complement most phenotypes associated with lack of Pho85, and Cdk5 associates with and is activated by Pho85 cyclins Pho80 and Pcl2 in yeast cells. The Pho85 likewise associates with Cdk5 activators p35 and p25 to form an active kinase complex in mammalian and insect cells (Huang et al, 1999). However, in the effort of searching for Cdk5 homologue in C. elegans, only ncc-1 gene encoded kinase is found to closely relate to human Cdk1, Cdk2 and Cdk3, which is required for M phase in meiotic as well as mitotic cell cycles (Boxem et al, 1999).

Cdk5 protein in most species has 292 amino acids and is structurally related among species. It forms a bilobal 3D-structure, which is typical in most protein kinases. The small lobe (N-lobe) is rich in beta-sheets and contains the glycine-rich motif (Glycine 11, 13 and 16) important for ATP binding. This lobe also contains the PSSALRE helix (Pro45-Leu55), which is a common feature in all Cdks and is crucial for interaction with interactive subunits. Three conserved residues, Lys33, Glu51 and Asp143 form a catalytic triad that helps orient ATP and facilitate catalysis. The bigger C-lobe is predominantly helical and contains the T-loop. This T-loop moves down from an inhibitory position to a fully extended configuration upon association with the regulatory proteins to allow the binding of the substrate and subsequent phosphorylation (Sharma et al, 1999).

Like the other Cdks, Cdk5 is a proline-directed kinase that phosphorylates serines or threonines immediately upstream of a proline residue. The consensus sequence in Cdk5 substrates is preferentially (S/T)PX(K/H/R), where S or T are the phosphorylated serine or threonine, X is any amino acid, P is the proline in the +1 position, and a basic residue (K or H or R) should be in the +3 position (Beaudette et al, 1993; Songyang et al, 1996). The list of Cdk5 substrates is growing (see Table 1 for substrates). Although there is overlapping of substrates between Cdk5 and other Cdks, no evidence has been found linking Cdk5 function to cell cycle progression, in which most other Cdks control the transition between different phases (Dhavan & Tsai review, 2001).

Originally demonstrated as an important player during neuronal development and differentiation, Cdk5 has been referred to as NCLK (neuronal Cdc2-like kinase) due to its great homology to human Cdc2 and high levels of expression in differentiated neurons

(Hellmich et al, 1992). Since tau, a neuronal microtubule-associated protein, is a substrate of Cdk5, Cdk5 is also named tau protein kinase II (Uchida et al, 1994; Sobue et al, 2000). To date, studies from several groups, including our lab, have illustrated that the activation of Cdk5 also plays an important role during cell death in both neuronal (for review, see Dhavan & Tsai, 2001) and non-neuronal systems (Ahuja et al, 1997; Gao et al, 1997; Zhang et al, 1997; Zhu et al, 2002; Zhu's thesis, 2002). In this thesis we are going to demonstrate a general correlation between Cdk5 activation and cell death and further evaluate how Cdk5 activation is regulated during cell death.

Table 1: Substrates of Cdk5.

Cdk5 Substate	Possible Functions of Phosphorylation	Refs.
P35	Promotes ubiquitin-mediated proteolysis	Patrick et al, 1998
P39		Humbert et al, 2000
PAK1	Inhibits PAK1 activity, regulation of actin dynamics	Nikolic et al, 1998
Src	Regulation of cell adhesion, actin dynamics, integrin signaling	Kato et al, 1999
Cables	Regulation of interaction of Cdk5 with c-Abl	Zukerberg et al, 1999
β-catenin	Regulation of cell adhesion, decreases association with presenilin 1	Kesavapany et la, 2001
Tau	Decreases binding to microtubules, and inhibits microtubule nucleation	Ishiguro et al, 1994
MAP1B	Regulation of microtubule stability	Paglini et al, 1998

(To be cont'd)

Table 1 (*cont'd*)

Nudel	Regulation of dynein-mediated axonal transport	Niethammer et al, 2000
NFH/NFM	Regulation of intermediate filament structure and transport	Grant et al, 2001
Synapsin 1	Regulation of synaptic transmission	Matsubara et al, 1996
MUNC18	Disrupts the Nunc18/syntaxin 1A complex, regulation of neurosecretion	Shuang et al, 1998
Amphyphysin1	Regulation of synaptic vesicle endocytosis	Floyd et al, 2001
β-APP	Regulation of APP localization, membrane transport	Lijima et al, 2000
DARPP32	Regulation of dopaminergic signaling	Bibb et al, 1999
PP1-inhibitor	Modulation of amplitude of cAMP-dependent signaling	Bibb et al, 2001
Pgamma (PDE regulator)	Regulation of retinal phototransduction	Hayashi et al, 2000
ERBB	Regulation of signaling at the neuromuscular junction	Fu et al, 2001
PRb	Neuronal differentiation and apoptosis	Lee et al, 1997
P53	Increases p53 transcriptional activity	Zhang et al, 2002
Pctaire1	Enhances Cdk5 kinase activity	Cheng et al, 2002
Canoe	*Drosophila* morphogenesis	Takahashi et al, 2002

B. Expression and activity of Cdk5 in cellular functions

Cdk5 mRNA has been detected in most mammlain tissues and various proliferating and differentiated cells (Meyerson et al, 1992; Tsai et al, 1993; for review, see Dhavan & Tsai, 2001). Among the different mammalian tissues, Cdk5 protein is expressed at the highest levels in neurons, at high levels in testis, and at relatively lower levels in ovaries and kidneys (Tsai et al, 1993). However, the kinase activity of Cdk5 has been associated only with differentiation and cell death (for review, see Lew & Wang, 1995; Ahuja et al, 1997; Fu et al, 2002; Zhu et al, 2002).

a. Expression and activity of Cdk5 in differentiation

i). *Expression and activity of Cdk5 in neuronal differentiation*

Cdk5 and its kinase activity play a crucial role during the development of the central nervous system (CNS). Studies of Cdk5 function during neuronal development have shed light on the involvement of Cdk5 in neurite migration, axon patterning, cortical lamination, neuronal secretion, neuronal adhesion, differentiation of oligodendrocytes, formation of synaptic structure and plasticity, and the maintenance of neuronal cytoarchitecture (for review, see Dhavan & Tsai, 2001). Unlike the other Cdks whose activities are mainly relevant with cell cycle regulation, Cdk5 kinase activity has been found predominantly in post-mitotic neurons (Tsai et al, 1993), though Cdk5 mRNA and protein are widely distributed in a variety of tissues and cell types.

During brain development, the expression and activity of Cdk5 is associated with the extent of neuronal differentiation (Tsai et al, 1993; Nikolic et al, 1996; Fu et al, 2002), whereas Cdc2 decreases at both protein and kinase activity levels during this process (Dobashi et al, 2000). The finding that Cdk5 is the only detectable Cdk in

hippocampal cell cultures further indicates that Cdk5 plays a different role in post-mitotic neurons than other Cdks in cell proliferation (Alvarez et al, 1999). During neuronal differentiation of NT2 cells (a human embryonal carcinoma cell line) following treatment with retinoic acid, the expression of Cdk5 is gradually increased and the Cdk5 kinase activity is induced (Fu et al, 2002). Dominant negative mutants of Cdk5 inhibit neurite outgrowth in primary rat cortical neurons (Nikolic et al, 1996). Cdk5 also helps maintain the stability of neuronal cytoskeletal network by stabilizing neurofilament (NF) protein network in neurons. NFs are the most extensively phosphorylated proteins in adult nervous systems, whose phosphorylation is important to regulate axonal transport and to affect the conduction velocity in neurons (Nixon & Sihag, 1991). It has been found that Cdk5 is a major NF-phosphorylating kinase in neurons (Shetty et al, 1992). The essential role of Cdk5 in the cytoarchitecture of CNS can be demonstrated by gene-targeting experiments. Mice deficient of Cdk5 die perinatally, and exhibit massive abnormalities, including lesions in the CNS, lack of cortical lamination and cerebellar foliation (Ohshima et al, 1996; 1999). The lethality of the Cdk5 null mice can be completely rescued by expressing the Cdk5 transgene in brain (Tanaka et al, 2001).

The kinase activity of Cdk5 requires association with its regulatory proteins, which are prominently p35 (Lew et al, 1994: Tsai et al, 1994) and p39 (Tang et al, 1995). Unlike Cdk5, the expression of p35 and p39 is more restricted, detected only in neurons (Tsai et al, 1994; Patzke & Tsai, 2002) and muscles (Lazaro et al, 1997; Fu et al, 2001). However, our lab has established an expression of p35 during cell death in non-neuronal tissues that correlates with Cdk5 activation (Ahuja et al, 1997; Gao et al, 1997; Zhu et al, 2002). Studies on different gene knockout mice have shown that p35 and p39 are

essential and sufficient for Cdk5 function involved in neuronal differentiation. P35 knockout mice show an inverted layering of cortical neurons similar to that seen in Cdk5-/- mice, but are viable and fertile with only mild disruptions in the hippocampus and a fairly normal cerebellum (Chae et al, 1997) compared to Cdk5-/- mice, where widespread disruptions are seen in neuronal layering of many brain structures, including the cerebral cortex, hippocampus, cerebellum and olfactory bulb (Ohshima et al, 1996; 1999). This discrepancy in the phenotypes of p35-/- and Cdk5-/- mice can be explained by the compensatory role of p39. The fact that p39 mutant mice do not show any outward phenotype argues that p35 can mask the absence of p39 (Ko et al, 2001). However, the phenotype of p35/p39 dual knockout mice is identical to that of Cdk5-/- mice (Ko et al, 2001), further indicating that p35 and p39 work together sufficiently for Cdk5 function.

ii). *Expression and activity of Cdk5 in non-neuronal differentiation*

Several studies have demonstrated that the expression and activity of Cdk5 are not restricted in neuronal tissues. Cdk5 has been documented to be a pivotal regulator of myogenesis. In *Xenopus*, Cdk5 is expressed in developing somites, and the dominant negative Cdk5 mutants suppress expression of the master regulators of myogenesis, MyoD and MRF4, resulting in disruption of somitic muscle patterning (Philpott et al, 1997). In murine fibroblast C2 cells, both the protein level and kinase activity of Cdk5 show a marked increase during early myogenesis. Microinjection with wild-type Cdk5 plasmids or dominant negative Cdk5 plasmids into C2 cells enforces or inhibits differentiation, respectively (Lazaro et al, 1997).

A functional link has been shown between Cdk5 activity and monocytic differentiation. The expression and activity levels of Cdk5 increase when HL-60 cells are

induced to differentiate to the mature monocytic phenotype upon exposure to 1, 25-dihydroxyvitamin D3 (Chen et al, 2000). Ectopic expression of Cdk5/p35 in undifferentiated HL-60 cells induces monocytic morphology, indicating a role of Cdk5 in haematopoietic cell differentiation (Chen & Studzinski, 2001).

Cdk5 and p35 are expressed in lens epithelial cells and in differentiating lens fibers during the development of rat lens, indicating a possible role of Cdk5 during differentiation of lens fiber cells (Gao et al, 1997). Cdk5 expression and kinase activity have been found in Leydig cells, Sertoli cells, spermatogonia and peritubular cells of developing rat testis (Musa et al, 1998; Session et al, 2001), suggesting that Cdk5 might play a role in spermatogenesis.

b. Expression and activity of Cdk5 in cell death

i). *Expression and activity of Cdk5 in neuronal cell death*

As discussed before, cell death is an essential component of development and homeostasis. It is also involved in pathologic situations, such as neuronal injury or neurodegeneration. Interestingly, Cdk5 activity is dramatically induced under these physiological or pathological conditions.

In developing mouse nervous system, between embryonic day 12 to 14, the massive cell death in the dorsal root ganglia and the trigeminal ganglia is accompanied by elevated levels of Cdk5 expression and its induced kinase activation (Zhang et al, 1997). In neurons of rat substantia nigra injected with hydroxydopamine and axotomy, Cdk5 protein as well as p35 protein is expressed exclusively in apoptotic profiles at late morphologic apoptotic stages (Neystat et al, 2001). In amyotrophic lateral sclerosis (ALS) mouse models, the deregulated increase of Cdk5 activity is associated with

hyperphosphorylation of tau and neurofilament proteins in affected dying motor neurons (Nguyen et al, 2001). In rat hippocampal neurons, β amyloid peptide (Aβ) treatment leads to cell death coinciding with a significant increase of the Cdk5 activity and tau hyperphosphorylation, which can be prevented by inhibiting Cdk5 activity using either Cdk5 chemical inhibitor butyrolactone I or Cdk5 anti-sense probes (Alvarez et al, 1999; 2001). Introduction of elevated Cdk5 activity leads to apoptosis in primary cortical neuron culture (Patrick et al, 1999; Lee et al, 2000). In differentiated PC12 cells, removal of nerve growth factor (NGF) induces apoptosis and increases Cdk5 expression and activity (Zhang & Johnson, 2000). In human neuroblastoma IMR-32 cells induced to die by oxidative stress, there is an upregulation of p35 and Cdk5 expression as well as Cdk5 activity in the affected cells, showing that the Cdk5 activity is involved in the signaling pathway of oxidative stress-related neurodegeneration (Strocchi et al, 2003).

Therefore, it seems that Cdk5 expression or activation is related to neuronal cell death.

ii). *Expression and activity of Cdk5 in non-neuronal cell death*

The association between Cdk5 expression/activity and cell death is not restricted to neurons. It appears that Cdk5 may be expressed or activated during cell death in different other systems, demonstrated by our lab. In the interdigital areas of developing mouse limbs, cell death shapes the contour of the limb with increased level of Cdk5 expression and dramatically induced Cdk5 activity (Ahuja et al, 1997). During nuclear death of lens fibers in rat developing lens, Cdk5 is highly expressed in the dying cells (Gao et al, 1997). In atrophic mouse ovarian follicles, Cdk5 is expressed in follicular cells undergoing apoptosis (Zhang et al, 1997). In regressing mouse prostate upon

androgen withdrawal, there is a dramatic increase of both Cdk5 expression and activity in the dying prostatic epithelium (Zhang et al, 1997). In retinoic acid-treated developing mouse limbs, marked levels of cell death are induced in the interdigital regions corresponding with an elevated Cdk5 expression and activation (Ahuja et al, 1997). In developing mouse embryos treated with cyclophosphamide, the increased level of Cdk5 expression and induced kinase activity accompanies cell death (Zhu et al, 2002).

Taken together, Cdk5 expression and activation are associated with cell death in both neuronal and different non-neuronal systems. In this thesis, we will further elucidate whether there is a general correlation between Cdk5 activation and cell death in a diversity of situations, ranging from normal cell death either during development or in adult to induced cell death in different pathologies.

C. Regulation of Cdk5 in cellular functions

The involvement of Cdk5 activity in many different facets of an organism's cellular activities, including cell differentiation and death, suggests that Cdk5 activation needs to be tightly regulated. This regulation has been demonstrated in several levels, such as binding to its regulators, transcriptional activation, and phosphorylation modification.

a. Regulation of Cdk5 in differentiation

i). *Association with the regulatory subunits: p35, p39*

Cdk5 kinase is activated by association with its regulatory subunits. There are two major neuronal Cdk5 activators, p35 (Tsai et al, 1994) and p39 (Tang et al, 1995), which are predominantly expressed in post-mitotic neurons of the CNS. Studies on the

spatial and temporal expression of p35 and p39 in rodent brains have shown a complementary pattern indicating that p35 and p39 activate Cdk5 at different stages and in different regions of both developing and adult brains (Cai et al, 1997). Further evidence has demonstrated that p35 and p39 localize to overlapping but distinct subcellular compartments in growth cones, synapses, and cytoskeleton as well as membrane (Humbert et al, 2000a & b; Niethammer et al, 2000).

Upon associating with its regulatory subunit, Cdk5 will form a fully active conformation from a bilobal structure to allow the binding of its substrate for subsequent phosphorylation (Tarricone et al, 2001). P35 is also a substrate of Cdk5, and after being phosphorylated by Cdk5, p35 undergoes degradation through a ubiquitin-proteasome pathway. Therefore, p35 is an unstable protein with a half-life of 20-30 minutes (Patrick et al, 1998). Inhibition of Cdk5 kinase activity confers longer half-life of p35 in primary neurons. P35 mutants without Cdk5 phosphorylation sites have increased stability (Patrick et al, 1998). This suggests a negative feed-back regulation of Cdk5 through p35. Although the finding that p39 is phosphorylated in Cdk5/p39 transfected COS-7 cells leads to the conclusion that p39 is a possible substrate of Cdk5 (Humbert et al, 2000), the function of p39 phosphosrylation still remains unclear.

As mentioned earlier, studies of the phenotype of different mutant mice (p35-/-, p39-/-, Cdk5-/-, and p35/p39 double knockout) have suggested that p35 and p39 are essential and sufficient Cdk5 regulators for neurodevelopment (Ohshima et al, 1996; 1999; Chae et al, 1997; Ko et al, 2001). However, is Cdk5 activated by the same regulators during cell death? We will discuss this in the section "Regulation of Cdk5 in cell death".

ii). *Transcriptional regulation*

The activation of Cdk5 can also be regulated at the transcriptional level during differentiation, via increasing the level of transcripts of either Cdk5 or p35. Different signaling pathways can increase the mRNA levels of p35 and Cdk5, leading to elevated activation of Cdk5. For instance, in cultured cerebellar macroneurons and SH-SY5Y neuroblastoma cells, the binding of laminin to integrin receptors triggers the transcription of p35 (Paglini et al, 1998; Li et al, 2000). The ERK (extracellular-signal-regulated kinase; also known as mitogen-activated protein kinase, MAPK) cascade has been shown to have a crucial role in cell proliferation and differentiation. In PC12 cells, sustained activation of ERK induced by NGF (nerve-growth factor) is essential for the differentiation of PC12 cells into sympathetic neurons. It has been found that Cdk5/p35 activity is required for this NGF-induced neuronal differentiation, and the sustained ERK activation is necessary and sufficient for the induction of p35 transcription through the induction of Egr1, an ERK-mediated transcription factor (Harada et al, 2001). In primary cultures of embryonic rat cortical neurons, addition of brain-derived neurotrophic factor (BDNF) leads to Cdk5 activation by induction of p35 transcript, which is inhibited by coincubation with either BDNF antibody or K252a, an inhibitor for BDNF receptor TrkB tyrosine kinase (Tokuoka et al, 2000). During chronic cocaine administration and repeated electroconvulsive seizure treatments (an effective treatment for depression), the transcription factor δFosB is induced in striatum or hippocampus and cerebral cortex. The physiological targets of δFosB include two glutamate receptor subunits: the NR1 subunit of NMDA receptors and the GluR2 subunit of AMPA receptors, which are both involved in dopamine signaling pathway. It has been shown that the induction of δFosB

also raises the expression of Cdk5 mRNA hence Cdk5 activity, indicating the involvement of dopamine pathway during Cdk5 activation (Greengard et al, 1999; Chen et al, 2000; Bibb et al, 2001).

Taken together, Cdk5 activation can be regulated at the transcriptional level during cell differentiation or other processes such as drug addiction and anti-depression treatment. Whether there exists this transcriptional regulation of Cdk5 activation during cell death remains to be explored, and will be studied in this thesis.

iii). *Phosphorylation of Cdk5*

The mitotic Cdks are regulated by three distinct phosphorylation events, including the inhibitory phosphorylation of Thr14 and Tyr15 and stimulatory phosphorylation of Thr160 (Gu et al, 1992; for review, see Dhavan & Tsai, 2001). Cdk5 protein itself can also be phosphorylated at three sites, Ser159, Thr14, and Tyr15. While phosphorylation of Thr160 in the T-loop of most other Cdks is required for their maximal activation, most researchers feel that phosphorylation of the equivalent Ser159 on Cdk5 is dispensable, mostly based on the study on the Cdk5-p25 (a truncated form of p35, often associated with deregulated Cdk5 activation during cell death) structure (Qi et al, 1995; Poon et al, 1997; Tarricone et al, 2001), although one group has shown that the phosphorylation of Ser159 on Cdk5 can increase Cdk5/p25 activity (Sharma et al, 1999). The structure of Cdk5-p25 shows that Ser159 in the T-loop of Cdk5 is in very close proximity to p25, and the addition of a phosphate group on Ser159 will adversely effect association with the activator. This indicates that phosphorylation of Ser159 of Cdk5, if occurring, will probably negatively regulate Cdk5 activity (Tarricone et al, 2001). Phosphorylation of Thr14 by a protein kinase purified from bovine thymus cytosol has been found to inhibit

Cdk5 activity (Matsuura et al, 1996). But c-Abl mediated phosphorylation of Tyr15 on Cdk5 stimulates Cdk5 activity, and this phosphorylation is enhanced by binding of a c-Abl adaptor protein Cables to Cdk5 (Zukerberg et al, 2000). Therefore, phosphorylation of Cdk5 protein at different sites is a putative mechanism to regulate Cdk5 activity during cellular differentiation. Identification of Cdk5 phosphorylation during cell death remains an intriguing task.

Taken together, Cdk5 activation can be regulated at three levels during cell differentiation: 1) association with its regulatory proteins, such as p35 and p39; 2) by increasing the mRNA levels of Cdk5 and p35; 3) being phosphorylated at different sites. In the following sections, we will discuss how Cdk5 activation is deregulated during cell death, and how the deregulated Cdk5 activity is associated with cell death.

b. Regulation of Cdk5 in cell death

Compared to studies in neuronal differentiation, the regulation of Cdk5 during cell death has been less studied and is now the focus of several groups including our lab; it is also the major interest of this thesis.

One possible mechanism of the regulation of Cdk5 during cell death has been proposed and demonstrated by Tsai et al (Patrick et al, 1999; Lee et al, 2000), suggesting that Cdk5 is activated by p25 resulted from calpain-mediated cleavage of p35 during cell death.

p25 is produced by different neurotoxic insults, such as hydrogen peroxide, glutamate, maitotoxin and ionomycin treatments of culture primary neurons (Kusakawa et al, 2000; Lee et al, 2000; Nath et al, 2000). All of these treatments are able to disrupt calcium homeostasis and dramatically increase the intracellular Ca^{2+} concentration, which

is enough to promote the cleavage of p35 to p25 and lead to Cdk5 activation. Generation of p25 following these treatments can be blocked by specific inhibitors (such as calpastatin) of calpain, a calcium-dependent cysteine protease, which has been discussed earlier in the Introduction. Furthermore, immunodepletion of calpain markedly reduces the conversion of p35 to p25 in these treatments. These results indicate that calpain may be the protease leading to p25 induction and hence Cdk5 activation during neuronal cell death.

P25 contains all the elements of p35 necessary for Cdk5 binding and it efficiently activates Cdk5 (Qi et al, 1995; Pooh et al, 1997). However, p25 has properties distinct from p35. First, p25 has a 5-10 fold longer life span than that of p35 (Patrick et al, 1999). Second, p25 lacks the amino-terminal myristoylation site and is mainly located in cytosol, while p35 is predominantly restricted to membrane area (Patrick et al, 1999). Therefore, the generation of p25 is likely to disrupt the normal regulation of Cdk5 by causing prolonged and mislocated Cdk5 activation.

p25 is neurotoxic. Co-transfection of Cdk5 and p25 induces apoptosis in primary cortical neurons, which exhibit neurite retraction and cytoskeletal abnormalities. This neuronal morphological degradation might result from Cdk5/p25-mediated hyperphosphorylation of neurofilaments and tau protein, since p25 regulated Cdk5 seems to phosphorylate its substrates more efficiently than p35 activated Cdk5 when p25 and p35 proteins are present at the same level (Patrick et al, 1999). Cdk5/p25 activation is involved in the increased phosphorylation level of tau in brains of Alzheimer's (AD) patients (Patrick et al, 1999; Gupta et al, 2001; Tseng et al, 2002). In transgenic mice overexpressing p25, a marked increase of Cdk5 activity corresponds to elevated levels of

tau and neurofilament phosphorylation, leading to disruption of neuronal cytoarchitecture, an event typical in AD and other neurodegenerative diseases (Ahlijanian et al, 2000). Aβ induces conversion of p35 to p25 in primary cortical neurons, and the inhibition of calpain and therefore blockage of p25 production protects neurons from cell death induced by Aβ (Lee et al, 2000). Town et al (Town et al, 2002) have also demonstrated that a soluble form of Aβ activates the Cdk5/p25 pathway and promotes AD-like tau phosphorylation in vitro. These observations suggest that Cdk5/p25 activity is related to the hyperphosphoralation of tau or neurofilament proteins in neuronal cell death.

Interestingly, in transfected neurons, Cdk5-p35 poorly phosphorylates tau (Patrick et al, 1999). Furthermore, there is no evidence for hyperphosphorylation of tau in triple transgenic mice expressing Cdk5, p35 and tau, despite elevated Cdk5 kinase activity and cell death (Van den Haute et al, 2001). Together, these findings argue that tau is not a physiological substrate of Cdk5/p35; under pathological conditions, p25 is produced allowing Cdk5/p25 to phosphorylate tau. This argument has been further confirmed by Takahashi et al's recent finding that tau is phosphorylated mainly by Cdk5/p39 during brain development. Takahashi's group found that the ability of Cdk5 to phosphorylate tau is much higher when in association with p39 than binding to p35, and tau phosphorylation at Ser202 and Thr205 is decreased in Cdk5-/- mouse brain but stays unchanged in p35-/- mouse brain. This suggests that Cdk5/p39 is responsible for the *in vivo* phosphorylation of tau at these sites, which are the major ones phosphorylated by Cdk5 in normal development (Takahashi et al, 2003).

Although p39 has been well studied as another major neuronal activator of Cdk5 during neuronal differentiation, its involvement in Cdk5 activation during cell death has been poorly understood. Until recently, one study has found that p39 can be cleaved to form a C-terminal fragment p29 by calpain in ischemic brain. This conversion of p39 to p29 results in a prolonged activation and misdistribution of Cdk5 in neurons associated with neuronal damage (Patzke & Tsai, 2002). This finding further supports the role of calpain in the regulation of Cdk5 activation during cell death.

Taken together, it has been shown that Cdk5 activity in association with p35 and p39 plays a key role in developing brain, and the abnormal activation of Cdk5 by calpain-mediated p25 production is closely involved in neuronal cell death. Therefore, a protective mechanism is needed to control Cdk5 activity in neuronal development. A recent study by Saito et al has found that the phosphorylation of p35 by Cdk5 suppresses its cleavage to p25 by calpain, whereas this phosphorylation facilitates the proteasomal degradation of p35 (Saito et al, 2003), which is consistent with Patrick et al's finding that phosphorylatioin of p35 by Cdk5/p35 leads to the degradation of p35 through a ubiquitin-proteasome pathway (Patrick et al, 1998). In this study, they also found that the phosphorylation site in p35 involved in preventing calpain cleavage is distinct from the phosphorylation site involved in facilitating proteasomal degradation. The phosphorylated form of p35 that is resistant to cleavage by calpain is more prevalent in the fetal brain, and the unphosphorylated form of p35 occurs in the adult brain. These results suggest that the phosphorylation of p35 serves as a protective mechanism that suppresses the generation of p25 in developing brain (Saito et al, 2003).

To date, the activation of Cdk5 by calpain-mediated p25 production has been

linked only to neuronal cell death. Whether this is a general regulation of Cdk5 activation during cell death has yet to be unfolded. To answer this question, in this thesis, we will evaluate whether Cdk5 is activated through the calpain-mediated p25 induction during cell death in systems other than neurons, including transformed mouse embryonic fibroblast cells exposed to different inducers of cell death and cyclophosphomide-treated mouse embryos.

PART II. MATERIALS AND METHODS

A. Materials

Animal treatment

Cyclophosphamide-treated mouse embryos

Swiss Webster mice (Charles River Laboratories, Wilmington, MA) were mated overnight, and early next morning females were checked for vaginal plugs. Positive females were designated as gestational day 0.5. On gestational day 9.5 females were injected intraperitoneally with 10mg/kg body weight of CP (Sigma, St. Louis, MO) dissolved in 0.9% saline. The treated mice were sacrificed by cervical dislocation 24 hrs after injection, and the embryos were removed.

p53 knockout mouse embryos

p53 heterozygous mice C57BL/6J-*Trp*53^{tm1Tyj} (Jackson Laboratories, Bar Harbor, ME) were mated overnight, and early next morning females were checked for vaginal plugs. Positive females were designated as gestational day 0.5 and the embryos were referred to embryonic day 0.5 (ED 0.5). On gestational day 13.5 females were sacrificed by cervical dislocation and the embryos were removed. Embryos were washed with PBS (Phosphate-Buffered Saline, 145.4 mM NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, 1.8mM KH_2PO_4, pH 7.4), and one hind limb was taken for genotyping. The rest of the embryos were fixed and embedded for sectioning as described below.

Caspase-3 knockout mouse embryos

Caspase-3 heterozygous mice (Gift from Dr. Richard Flavell, Yale University)

were mated overnight, and females with vaginal plugs were designated as gestational day 0.5. By day 13.5, pregnant female mice were sacrificed by cervical dislocation, and the embryos were removed. Embryos were washed with PBS, and one hind limb was taken for genotyping. The rest of the embryos were fixed and embedded for sectioning as described below.

Tissue, slide preparation and microscopy

Embryos were washed with PBS, fixed in 4% paraformaldehyde with shaking at 4°C for 18-24 hrs, and partially dehydrated in 20% sucrose solution in PBS for 2 days. Embryos were then embedded in OCT compound (Miles, Elkhart, IN) for sectioning at 5 μm.

Sections of rat lungs infected with *Streptococcus sanguils* or *Streptococcus pneumoniae* type 25 were from Dr. Jeffrey Kazzaz (Winthrop-University Hospital; see Kazzaz et al, 2000). Sections of mouse limb infected with *Leishmania major* were from Dr. Karen Newell (University of Colorado at Colorado Springs; see Desbarats et al, 2000). Sections of preterm lamb lung after gas and liquid ventilation were from Dr. Lin Mantell (North Shore Hospital Institute; see Mantell et al, 2002). Slides for Apaf-1-deficient mouse embryos were a gift from Dr. F Cecconi (Germany).

Antibodies and reagents

Chemical reagents were ordered from Sigma (St. Louis, MO) unless otherwise mentioned. Roscovitine was purchased from BIOMOL Research Laboratories Inc. (Plymouth Meeting, PA). The calpain inhibitors, calpastatin peptide (Cat# 208902) and

PD150606 (Cat# 513022), were obtained from CalBiochem (La Jolla, CA). The antibodies used in this paper include anti-Cdk2 (M-2), anti-Cdk3 (Y-20), anti-Cdk4 (H-303), anti-Cdk5 (C-8), anti-Cdk6 (C-21), anti-p35 (C-19), anti-PARP (H-250) antibodies from Santa Cruz Biotechnology (Santa Cruz, CA), anti-active caspase 3 antibody from BD PharMingen (San Diego, CA), and anti-spectrin antibody from Chemicon International (Temecula, CA).

Cell culture and treatment

C8 and A9 cells are mouse embryonic fibroblast cell line transformed with E1A and ras, with C8 p53+/+ and A9 p53-/- (Gift from Dr. Scott Lowe, Cold Spring Harbor Laboratory). Cells were maintained in DMEM (Dulbecco's Modified Eagle Medium, Life Technologies, Rockville, MD) supplemented with 10% fetal bovine serum, 50U/ml penicillin and 100μg/ml streptomycin, at 37°C in a humidified atmosphere of 7.5% CO_2. At 80% confluence, cells were treated in DMEM containing 1% FBS with different cytotoxic reagents, such as 2.5% ethanol (EtOH), 25μg/ml cycloheximide (CHX, dissolved in ethanol), and 20μM camptothecin (CPT, dissolved in DMSO), for 18 hrs. For the kinetic study of cells treated with 20μM CPT or irradiated with 254 nm ultraviolet (UV, 50 J/m^2), cells were collected at different time points (i.e. 0hr, 3hr, 6hr, 8hr, 12hr, 15hr, and 18hr for CPT, and 0hr, 8hr, 12hr, and 18hr for UV). To block calpain activation induced by CPT in both C8 and A9 cells, two different calpain inhibitors, 10 μM calpastatin (CS) or 10 μM PD150606 were coadministrated with CPT and cells were incubated for 18 hrs. In the experiment testing the effect of inhibition of Cdk5 activation by roscovitine on CPT-induced cell death in C8 and A9, 10 μM

roscovitine was added to media with or without CPT (20 μM).

Primary mouse embryonic fibroblast cells (wild type, caspase-3-/-, and caspase-9-/-, and Apaf-1-/-; all gifts from Dr. Lowe, CSHL) were maintained in DMEM (Dulbecco's Modified Eagle Medium, Life Technologies, Rockville, MD) supplemented with 10% fetal bovine serum, 50U/ml penicillin and 100μg/ml streptomycin, at 37°C in a humidified atmosphere of 5% CO_2. At 80% confluence, cells were treated in DMEM containing 1% FBS with different cytotoxic reagents, such as 25 or 50 μg/ml cycloheximide (CHX, dissolved in ethanol), and 20 or 25 μM camptothecin (CPT, dissolved in DMSO), for 18 hrs.

Cell death was measured by trypan blue dye exclusion. Proteins were extracted from cells after treatment for western blot.

B. Cellular and Molecular Methods

DNA fragmentation assay

DNA fragmentation was detected by using TUNEL POD (Terminal deoxynucleotidyl transferase-mediated d-UTP Nick End Labeling Peroxidase) combined with nonisotopic digoxigenin-11-dUTP and terminal transferase according to manufacturer's instruction (Roche Molecular Biochemicals, Germany). Briefly, slides were incubated with permeabilization solution (0.1% TritonX-100 in 0.1% sodium citrate) on ice for 2 min, and endogenous peroxidase activity was quenched with 0.1% hydrogen peroxide in methanol at RT for 30 min. After 2 rinses in PBS, TUNEL reaction mixture (9 volume of TUNEL label and 1 volume of TUNEL enzyme) was applied to

slides, which were incubated for 30 min at 37°C followed by three washes with PBS. TUNEL POD was then applied to the slides to bind to the FITC-dUTP enzymatically added to the free end of the oligonucleotide and visualized with DAB (diaminobenzidine, Research Genetics, Huntsville, AL). The sections were counterstained with methylene blue and mounted with Permount® (Fisher Scientific, Burr Ridge, IL).

Immunohistochemistry

In situ protein level of different genes was detected using the ABC (Avidin-Biotinylated-peroxidase Complex) kit (Vectastain ABC kit, Vector Laboratory, Burlingame, CA). Sections were quenched with 0.3% hydrogen peroxide in methanol at RT for 20 min to abolish endogenous peroxidase activity. After three washes with PBST (0.1% Tween 20 in PBS), sections were incubated in blocking solution at RT for 1 hr, and treated with primary antibody (Cdk5 and p35: 0.2 μg/ml; active caspase 3: 0.5 μg/ml) at 4°C in a humidified chamber overnight. Following three washes with PBST, secondary biotinylated antibody was applied to the sections for 1 hr at RT. Sections were then washed with PBST for three times and incubated with ABC reagent for 2 hrs at RT. Sections were washed with PBST for three times before being visualized with DAB, counterstained with methylene blue, and mounted with Permount.

DNA fragmentation and immunohistochemistry double labeling

Fluorescence detection of DNA fragmentation and Cdk5 expression were used as in Ahuja et al (1997). Briefly, sections were twice washed in PBS, postfixed in ethanol: acetic acid (2:1) for 5 min at -20° C, and again twice washed. Sections were placed for 5

min in equilibration buffer (Apoptag® kit, Intergen, Purchase, NY), and the solution was changed for one containing TdT and the incubation was continued for 1 hr at 37°C. The slides were immersed in pre-warmed stop-wash buffer for 30 min, rinsed three times with PBS, and exposed to anti-digoxigenin FITC fluorescein and incubated for 30 min. After a wash with PBST, slides were incubated for 20 min in 0.3% H_2O_2, followed by two washes with PBST. Primary anti-Cdk5 antibody was added to blocking solution (1 mg/mL bovine serum albumin in PBST) to a final concentration of 1 μg/mL. The incubation in primary antibody was overnight at 4° C. The sections were washed three times with PBST, incubated with secondary biotinylated antibody overnight at RT, and again rinsed three times. Fluorescent signal was added by incubating the slides with cy3-conjugated IgG mouse anti-biotin (Jackson Immuno Research Laboratory, West Grove, PA) for 30 min. The slides were mounted with 90% glycerol and observed under an Ultima 312™ confocal microscope (Meridian International, Inc., Okemos, MI). DNA fragmentation is seen with FITC as green and the Cdk5 as detected by cy3 is red.

Probe preparation

Cdk5 (270 bp insert BamHI/EcoRV) cDNA was gift of Dr. P. Zalenka (NIH). For preparation of radioactive RNA probes, DNA template was incubated with transcription solution (1X transcription buffer, 10 mM DTT, 0.5 mM CPT, 0.5 mM GTP, 50 mCi ^{35}S-UTP, and RNAsin, RNA polymerase) for 2 hrs at 37 °C. More polymerase was added with a further incubation for 30 min, followed by the addition of RNase free DNase RO1 (Promega, Madison, WI) and incubated for 15 min. After the addition of total yeast RNA, solution A (10 mM DTT, 80 mM $NaHCO_3$, 120 mM Na_2CO_3) was added to the digest at

60° C, followed by the addition of solution B (200 mM NaOAc, 10 mM HOAc, 10 mM DTT). The probes were purified on G-50 Sephadex column, ethanol precipitated and dissolved in 1 M DTT to use at final concentration of 25, 000-50,000 CPM/μL in hybridization buffer (50% formamide, 0.3 M NaCl, 20 mM Tris-HCl (pH 7.4), 5 mM EDTA, 10 mM $NaH_2PO_4 \cdot H_2O$ (pH 8.0), 10% dextran sulfate, 1X Denhardt's solution, 0.5 mg/mL total yeast RNA).

In situ hybridization

The *in situ* hybridization was as described previously (Ahuja et al, 1997). Briefly, paraformaldehyde postfixed sections were incubated with proteinase K in PK buffer (1 M Tris, 0.5 M EDTA). After dehydration in graded ethanol, sections were hybridized with hybridization buffer and either Cdk5 or p35 probe in 10 mM DTT. Sections were then washed in 5X SSC with 10 mM DTT, in 50% formamide in 2X SSC, in 1X washing solution (23.4 g NaCl, 10 mL 1 M Tris (pH7.5) with 5 mM EDTA, in 20 mg/mL RNase A in 1X washing solution, in 1X washing solution, in 2X SSC, and 0.1X SSC. Finally, the dehydrated sections were dipped in photographic NTB-2 emulsion (Kodak) and exposed for 1-2 weeks, after which they were developed (Kodak Dektol developer), fixed (Kodak), counterstained with 0.2% toluidine blue, dehydrated, and mounted with Permount.

Northern blot

Total RNA was extracted from mouse embryos using ice-cold lysis buffer (4 M guanidinium isothiocyanate, 25 mM sodium citrate pH 7.0, 0.5% Sarkosyl®, and 0.1 M

β-mercaptoethanol), with the addition of 2 M sodium acetate (pH 4.0) and phenol as well as chloroform:isoamylalcohol (49:1), in succession. Upon precipitation with isopropanol, the RNA pellet was resuspended in lysis buffer, and precipitated again in 75% ethanol and recovered by centrifugation. After dissolving RNA in sterile dH_2O, we determined the concentration of RNA by measuring the absorbance at 260 nm and the A_{260}:A_{280} ratio. Collected total RNAs were run on a 1% agarose gel and the separated bands were transferred to a nitrocellulose membrane. RNAs immobilized on the nitrocellulose were hybridized with a solution containing ^{32}P-labeled RNA probe. The nonhybridized radioactive RNA probe was washed, and the hybridization was analyzed by autoradiograph.

Western blot analysis

Cells or tissues were lysed in RIPA buffer (50 mM Tris, pH7.5, 150 mM NaCl, 1% Triton 100X, 0.1% SDS, 0.5% sodium deoxycholate, 2 mM EDTA, and protease inhibitor cocktail tablets (Boehringer Mannheim, Germany)) and cell/tissue debris were rid by centrifugation at 13,000 rpm for 30min at 4°C. Protein concentration was determined using the Bio-Rad microassay (Bio-Rad laboratories, Hercules, CA). Equal amounts of protein were run on 8%, 12.5% or 15% SDS-polyacrylamide gels after addition of equal volume of 2X Laemmli loading buffer (100mM Tris, pH 7.5, 4% SDS, 20% glycerol, 0.002% bromophenol blue) (Laemmli, 1970). After protein transfer, nitrocellulose membrane blots were blocked with 5% non-fat dry milk in PBST for 30min and incubated with primary antibody (0.5μg/ml for Cdk2-6 or p35 or caspase 3, 0.1μg/ml spectrin and PARP) on the shaker overnight at 4°C. After three washes with

PBST, blots were incubated with horseradish peroxidase (HRP) conjugated goat anti-rabbit antibody (Jackson Immuno Research Laboratory, West Grove, PA) for 1 hr at RT followed by 3 washes. The immune complexes were detected by chemiluminescence (ECL plus kit, Amersham, Chicago, IL) and exposed to autoradiographic film.

In vitro histone H1 kinase assays

Cdk5 kinase activity was assayed as described by Zhang et al (1997). Equal amounts of lysates from control and CP embryos or different cell samples were incubated with 1.5 μg/ml Cdk5 antibody for 1 hr at 4° C, and then purified by the addition of protein A-Sepharose (Boehringer Mannheim, Germany). The precipitated beads were equilibrated in kinase buffer (50 mM Tris, pH7.5, 10 mM $MgCl_2$, 1 mM DTT, 20 mM EGTA, 0.1 mM sodium vanadate, 80 mM β-glycerophosphate) and collected by centrifugation. Histone H1 kinase assays were performed in kinase buffer supplemented with 0.1μCi/ml [γ-^{32}P]ATP (6000 Ci/mM) (Amersham Pharmacia Biotech., Piscataway, NJ), 10 mM ATP, 50 μg/ml calf thymus histone H1 (Boehringer Mannheim, Germany), and 5 μM cAMP-dependent protein kinase inhibitor (Sigma, St. Louis, MO) at 30° C for 30 min. An equal volume of 2X Laemmli buffer was added to each sample before they were denatured at 100° C for 2 min. Following electrophoresis, gels were fixed, dried, and exposed to film. The intensity of the bands was quantified using a Storm Imaging System (Molecular Dynamics Inc., Sunnyvale, CA).

De novo protein synthesis ^{35}S-labeling

ED10.5 embryos were removed from pregnant female mice and placed in DMEM

(Dulbecco's Modified Eagle Medium, Life Technologies, Grand Island, NY) culture medium supplemented with 30% FBS (fetal bovine serum, Life Technologies), 50 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies), and 100 μCi/ml ^{35}S methionine and cysteine (Amersham Pharmacia Biotech., Piscataway, NY), on a shaker, at 37° C with 5% CO_2, for 3 hrs. Embryonic viability was ascertained by beating of the heart. During the 3 hr incubation, we found all of the embryos to be viable. The embryos were washed with PBS several times before being lysed in RIPA buffer. The lysates of embryos were stored at -20° C for both western blot and immunoprecipitation.

Cell death by trypan blue

The loss of membrane integrity exhibited in dead and dying cells allows the preferential uptake of vital dye trypan blue (Karasavvas et al, 1996). At the end of the treatment, cells were pelleted and washed with PBS. 100μl of well suspended cells was mixed with 100μl of 0.4% trypan blue solution (in PBS), and left at room temperature (RT) for 5 min. Cells were then viewed under a light microscope and blue stained cells were non-viable.

DNA isolation and analysis of fragmentation via agarose gel electrophoresis

Cells were lysed in lysis buffer (0.2% TritonX-100, 10 mM Tris/HCl 10 mM EDTA, pH7.5). The cell lysate was incubated on ice for 15 min followed by centrifuging at 4 °C at 13,000 rpm for 30 min. The supernatant containing the low-molecular-mass DNA was incubated with RNase (100 μg/ml) for 1 hr and then extracted twice with equal volume of phenol/chloroform/isoamyl alcohol (24:24:1) and once with

chloroform/isoamyl alcohol (24:1). The extracted low-molecular-mass DNA was precipitated with 300 mM NaCl and 2.5 volume of ethnol at –20 °C overnight and spun at 13,000 rpm for 30 min at 4 °C. The pellet was washed with 70% ethanol once, air dried, and suspended in 10 mM Tris/HCl, 1 mM EDTA, pH8.0. Low-molecular-mass DNA from equal numbers of cells was run on 2% agarose gel. Separated DNA bands were visualized by ethidium bromide staining.

Condensed nuclei stained by bis-benzimide

The DNA fluorochrome bis-benzimide (Hoechst 33258, Sigma, St. Louis, MO) was used to stain fragmented nuclei of apoptotic cells. Briefly, cells were trypsinized, pelleted and washed once with ice cold PBS. The cells were then resuspended and fixed in 3% paraformaldehyde solution (in PBS) for 30 min, washed with ice cold PBS, and incubated with 16μg/ml bis-benzimide (in PBS) for 25 min at RT. Finally, the cells were washed with ice cold PBS once and resuspended in PBS. 20μL of the cell suspension was evenly distributed on a slide and coverslipped to be viewed under an Eclipse TE300 microscope (Nikon Inc., Melville, NY).

Retroviral transfection of sense and anti-sense p35

The construct used to produce retroviral recombinants was based on the retroviral vector pJim. A pJim-IRES2-EGFP vector was made using NotI/BglII fragment form the pIRES2-EGFP expression vector (Clontech, Cat# 6029-1) containing an internal ribosomal entry site and EGFP to replace the NotI/BglII fragment containing EGFP in pJim. Briefly, XhoI/EcoRI fragment of p35-wt from pcDNA3-p35 was cloned into the

XhoI/EcoRI or EcoRI/XhoI site of pJim-IRES-EGFP, resulting in a sense p35 or anti-sense p35 recombinant, respectively. The transient retroviral production using 293T cells was adapted from Dr. G. Nabel's lab (NIH; Yang et al, 1999). Infection of C8 or A9 cells was carried by incubating the cells with viral supernatant after filtering through a 0.45 μm protein low-affinity filter (Fisher Scientific, Pittsburgh, PA) overnight, followed by culturing the cells with normal growth media for 36 hrs. Routinely, about 80%-90% cells showed fluorescence by 48 hrs after transfection. After this, the cells were ready to be exposed to different insults.

PCR genotyping of p53 knockout mice

Genotyping of embryos was performed according to the protocol recommended by Jackson Laboratories (http://www.jax.org/resources/documents/imr/supp_proto.html). Two sets of primers were used: 5'- CTT GGG TGG AGA GGC TAT TC –3' and 5'- AGG TGA GAT GAC AGG AGA TC –3'; 5'- ATA GGT CGG CGG TTC AT –3' and 5'- CCC GAG TAT CTG GAA GAC AG –3'. Briefly, the hind limb was lysed in 170 μl of Lysis Solution (50mM Tris, pH8.3, 100mM NaCl, 5mM EDTA, 0.8% SDS) and 30 μl of Proteinase K solution (10mg/ml) at 55°C for 5 hrs or overnight. After centrifuging at 13,000rpm for 15min, the supernatant containing DNA was precipitated using same volume of 100% isopropyl alcohol. DNA pellet obtained by centrifuging at 13,000rpm for 15min was dissolved in autoclaved ddH_2O and stored at -20°C. 10 to 200 ng of the extracted DNA was used for PCR. This PCR assay applied the "touchingdown cycling" technique. For the first 12 cycles, the annealing temperature, which started at 64°C, was reduced by 0.5 degree per cycle. For the remaining 25 cycles, the annealing temperature

stayed at 58°C. The PCR products were separated by gel electrophoresis on a 1.5% agarose gel. Wild type gave a band at 600bp, and p53 knockout showed a band at 280bp.

PCR genotyping of caspase-3 knockout mice

Three primers were used: 5'- CTT GGG TGG AGA GGC TAT TC –3' and 5'- AGG TGA GAT GAC AGG AGA TC –3'; 5'- ATA GGT CGG CGG TTC AT –3' and 5'- CCC GAG TAT CTG GAA GAC AG –3'. Briefly, either the hind limb of an embryo or the 0.5cm of the tail from an adult was lysed in 170 µl of Lysis Solution (50mM Tris, pH8.3, 100mM NaCl, 5mM EDTA, 0.8% SDS) and 30 µl of Proteinase K solution (10mg/ml) at 55°C for 5 hrs or overnight. After centrifuging at 13,000rpm for 15min, the supernatant containing DNA was precipitated using same volume of 100% isopropyl alcohol. DNA pellet obtained by centrifuging at 13,000 rpm for 15min was dissolved in autoclaved ddH_2O and stored at -20°C. 10 to 200 ng of the extracted DNA was used for PCR. This PCR assay applied the "touchingdown cycling". For the first 12 cycles, the annealing temperature which started at 64°C was reduced by 0.5 degree per cycle. For the remaining 25 cycles, the annealing temperature stayed at 58°C. The PCR products were separated by gel electrophoresis on a 1.5% agarose gel. Wild type gave a band at 320bp, and caspase-3 knockout showed a band at 300bp.

PART III. RESULTS AND DISCUSSION

Chapter I. Association of Cdk5 with cell death in pathological situations

A. Analysis of association of Cdk5 with cell death in rat lungs suffering from *streptococcus pneumoniae* type 25-caused pneumonia

B. Analysis of association of Cdk5 with cell death in *Leishmania major* -infected mouse limbs

C. Analysis of association of Cdk5 with cell death in preterm lamb lungs after gas or liquid ventilation

D. Analysis of association of Cdk5 with cell death in cyclophosphamide -treated mouse embryos

Chapter II. Up-regulation of Cdk5 during cell death

A. Analysis of Cdk5 up-regulation during cell death at transcriptional level

B. Analysis of Cdk5 up-regulation during cell death at translational level

C. Analysis of Cdk5 up-regulation during cell death at post-translational level

Chapter III. Dependency of regulation of Cdk5 activation on p53 function during cell death

A. Analysis of cell death induced by different stimuli in p53+/+ and p53-/- cells

B. Analysis of expression and activation of Cdk5 during cell death in

p53+/+ and p53-/- systems

C. Analysis of regulation of Cdk5 activation during cell death in p53+/+ and p53-/- cells

D. Analysis of kinetics of cell death induction in p53+/+ and p53-/- cells

Chapter IV. Dependency of Cdk5 activation on caspase activity during cell death

A. Dependency of Cdk5 activation on Apaf-1 and caspase-9 activity during cell death

B. Dependency of Cdk5 activation on caspase-3 activity during cell death

C. Dependency of Cdk5 activation on caspase cascade during cell death

OBJECTIVE

Previous findings from our lab and other groups have suggested an association between Cdk5 activation and cell death during development (Nikolic et al, 1996; Ahuja et al, 1997; Gao et al, 1997; Philpott et al, 1997; Pigino et al, 1997, 1998; Zhang et al, 1997; Musa et al, 1998, 2000; Session et al, 2001), adult homeostatic maintenance of various tissues (Zhang et al, 1997; Lilja et al, 2001), and in some pathogenic situations such as neurodegeneration and neuronal injuries (Patrick et al, 1999, 2001; Lee et al, 2000; Nath et al, 2000; Bu et al, 2002; Hashiguchi et al, 2002; Kusadawa et al, 2002; Sisodiya et al, 2002). However, there is very limited study on the correlation between Cdk5 and cell death in other pathological situations, such as infection and treatment involved in clinical therapy, including ventilation assistance and chemotherapy.

We therefore asked the question: is there a general association between Cdk5 and pathological cell death? To answer this question, we used different systems, including rat lungs suffering from *Streptococcus pneumoniae* type 25-caused pneumonia, mouse limbs infected with *Leismania major*, preterm lamb lungs after liquid or gas ventilation, and cyclophosphamide (CP)-treated mouse embryos. There are dramatic levels of cell death in these models resulting from the pathology, which makes them suitable to study the link between Cdk5 expression and cell death under pathological condition.

Establishing the association between Cdk5 and pathological cell death further suggests there is a general correlation between Cdk5 and cell death in various situations. The fact that different cell death pathways are possibly involved in these various situations indicates that many signaling pathways may converge to Cdk5 activation,

suggesting a downstream Cdk5-involved signaling pathway during cell death. As a kinase, Cdk5 activation can be regulated at different levels, including transcription, translation and post-translation. We therefore asked at what level Cdk5 was regulated during cell death. We used CP-treated mouse embryos as the model in an attempt to answer this question. In light of the fact that unlike other Cdks Cdk5 does not play any role in cell cycle progression, we further examined the uniqueness of Cdk5 correlation with cell death among Cdks, using CP-treated mouse embryos as the model. Previously, our lab has shown that Cdk5 is the only Cdk that is associated with cell death in developing mouse limb (Ahuja et al, 1997).

As discussed in the Introduction, cell death is a very complex process, comprised of many different signaling pathways working either independently or coordinately to ensure the execution of a doomed cell. The correlation between Cdk5 activation and cell death in different models, in which different cell death pathways might be involved, implicates Cdk5 activation as a very fundamental event in cell death. We therefore examined the relationship between Cdk5 regulation and other signaling pathways during cell death.

As illustrated earlier in the Introduction, p53 signaling pathway, a well-known pathway involved in cell death, plays a crucial role in the induction of apoptosis (Lowe et al., 1993; Clarke et al., 1993; for review, see Hickman et al, 2002). Evidence from a number of labs indicates that activation of p53 is essential for neuronal apoptosis during brain development (Nishino et al, 1995; Aloyz et al, 1998). The requirement for p53 activity and Cdk5 activity during neuron development therefore has let us to ask the relationship between Cdk5 activation and p53 pathway during cell death. Recently,

Zhang and colleagues found that in apoptotic differentiated PC12 cells the p53 level increases concomitantly with levels of Cdk5, and increased Cdk5 activity significantly elevates p53 transcriptional activity, levels of p53 as well as the p53-responsive genes, such as p21 and Bax (Zhang et al, 2002). P53 has also been implicated as a substrate of Cdk5 by *in vitro* study (Zhang et al, 2002). However, the role of p53 function in Cdk5 activation during cell death remains elusive. Our study presented in this thesis was to examine the dependency of Cdk5 activation on p53 during cell death using both *in vitro* cell lines and *in vivo* mouse embryos as model systems.

Also as discussed in the Introduction, proteases are important components of cell death machinery, and proteolytic cleavages play a key role in cell death. Caspases are among the most important and most widely studied proteases involved in cell death. During cell death, both initiator and executioner caspases are activated by either auto-processing or other caspase-mediated proteolysis in a process called caspase cascade. Although different signaling pathways are activated in response to different stimuli, they always converge to a common mechanism leading to a cell's demise, which is activation of caspases. Whether caspase activation is involved in this Cdk5-involved pathway has yet to be revealed. We therefore evaluated the dependency of Cdk5 activation on caspase-3, -9 or Apaf-1 during cell death, due to their important roles in caspase cascade. Both *in vitro* cell lines and and *in vivo* knockout mouse embryos were utilized as models.

Chapter I. Association of Cdk5 with cell death in pathological situations

A. Analysis of association of Cdk5 with cell death in rat lungs suffering from *Streptococcus pneumoniae* type 25-caused pneumonia

B. Analysis of association of Cdk5 with cell death in *Leishmania major* -infected mouse limbs

C. Analysis of association of Cdk5 with cell death in preterm lamb lungs after gas or liquid ventilation

D. Analysis of association of Cdk5 with cell death in cyclophosphamide -treated mouse embryos

1. Objective:

Studies from our lab and other groups have demonstrated that Cdk5 activity is involved in different aspects of an organism's life, from the normal development to cell death involved in tissues during development and homeostatic maintenance as well as in pathological situations. Findings such as that Cdk5 is the only detectable Cdk in hippocampal cell cultures (Alvarez et al, 1999), its kinase activity has been detected predominantly in post-mitotic neurons (Tsai et al, 1993), and the expression and activity of Cdk5 is associated with neuronal differentiation (Tsai et al, 1993; Nikolic et al, 1996; Fu et al, 2002), suggest an essential role of Cdk5 for development of the CNS. It has also been shown that Cdk5 plays an important role in the development of other tissues than the CNS as presented in the Introduction.

Our lab is one of the first groups to demonstrate that there exists a correlation between Cdk5 and cell death in a variety of systems. Activation of Cdk5 is found to associate with cell death during development, such as in mouse brain (Zhang et al, 1997), in the interdigital region of mouse limb (Ahuja et al, 1997), and in rat lens (Gao et al, 1997). Cdk5 activity is also associated with cell death occurring in adult tissues, such as in atrophic mouse ovarian follicles and in regressing mouse prostate upon estrogen withdrawal (Zhang et al, 1997). Furthermore, abnormal Cdk5 activation has been correlated with cell death involved in neuronal injuries (Nath et al, 2000) and neurodegenerative disorders, such as atrophic lateral sclerosis (ALS, Nguyen et al, 2001) and Alzheimer's disease (AD, Ahlijanian et al, 2000; Lee et al, 2000; Town et al, 2002).

Although the correlation between Cdk5 and cell death has been well studied in normal situations, i.e. during development and adult homeostatic maintenance of

different tissues, there are few reports on Cdk5 activation in pathological situations other than neurodegeneration. We therefore explored the association between Cdk5 and pathological cell death in different models, including rat lungs suffering from *streptococcus pneumoniae* type 25-caused pneumonia, mouse limbs infected with *Leishmania major*, preterm lamb lungs after liquid or gas ventilation, and cyclophosphamide (CP)-treated mouse embryos. Dramatic levels of cell death induced in these model systems resulting from different pathological situations make these model systems suitable to study the correlation between Cdk5 expression and cell death under pathological conditions.

In the light of the fact that Cdk5 is the only Cdk whose expression is associated with cell death in developing mouse limb (Ahuja et al, 1997), we also examined the uniqueness of Cdk5 expression in associated with cell death in CP-treated mouse embryos.

We found that Cdk5 is associated with cell death in pathological situations and its correlation with cell death is unique among Cdks.

2. Results:

A. Analysis of association of Cdk5 with cell death in rat lungs infected with *Streptococcus pneumoniae* type 25

Bacterial infection generally leads to cell injury and cell death within the affected organ. It has been reported that apoptosis of lung cells is a prominent component of acute lung injury, and also occurs within hours of initial infection in the following experimental models of pneumonia (Mantell et al, 1997). Infection with *Streptococcus pneumoniae* type 25 causes acute pneumonitis in rats. Using this model system, we examined cell death by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and Cdk5 expression by immunohistochemistry.

Pathogen-free Wistar rats were infected with *S. pneumoniae* type 25 and lung tissues were fixed, embedded, and sectioned as described in Materials and Methods. TUNEL-Cdk5 double labeling assay was performed on lung tissue sections taken 4 d after infection, since this time had the highest amount of cell death detected by image analysis (data not shown). Examination using confocal microscopy showed that the green fluorescent TUNEL-signaling (FITC) labeled a large number of cells with fragmented DNA in infected lung tissue (Fig. 1A), indicating cell death was markedly induced in rat lungs with acute pneumonitis. The red fluorescent dye (Cy3) labeled only cells expressing Cdk5 protein (Fig. 1B). Comparison of the TUNEL assay and Cdk5 staining results suggested that most TUNEL-positive cells seemed to be Cdk5 positive as well. To investigate whether Cdk5 expression was colocalized in the dying cells during bacterial infection, the respective images were superimposed, and an overlap between DNA fragmentation and the expression of Cdk5 was found in most

cells, as indicated by the intense yellow fluorescence (Fig. 1C). Therefore, we conclude that infection causes cell death and Cdk5 expression is correlated with cell death in rat lungs during bacterial infection. The data from this study in collaboration with Dr. Kazzaz have been published (Kazzaz et al, 2000).

B. Analysis of association of Cdk5 with cell death in *Leishmania major*-infected mouse limb

Leishmania major (Lm) is an intracellular protozoan parasite that causes spontaneously resolving cutaneous leishmaniasis in humans. Lm infection in mice is a prototypical model for the role of immune deviation in disease resistance. Female mice were infected with Lm and infected hind footpad was obtained, fixed, embedded, and sectioned as described in Materials and Methods. We examined *in situ* cell death in the draining lymph node of Lm infected mice by TUNEL assay. TUNEL staining showed that many cells in these regions were dying by apoptosis, indicated by the presence of fragmented DNA in dying cells (Fig. 2A & B). Immunohistochemistry using anti-Cdk5 antibody exhibited cells expressing Cdk5 in the lymph node of Lm-infected mice (Fig. 2C & D). Comparison of the dramatic increase of Cdk5 protein level and appearance of apoptotic cells presenting fragmented DNA in the draining lymph node of Lm-infected animals, suggests that Cdk5 expression is correlated with cell death in this Lm infection model system. The data from this section in collaboration with Dr. Newell have been published (Desbarats et al, 2000).

C. Analysis of association of Cdk5 with cell death in preterm lamb lung after gas or liquid ventilation

The survival of preterm infants requires prolonged ventilation assistance. The conventional gas ventilation (GV) leads to significant pulmonary injury resulting from induced cell death, due to prolonged exposure to hyperoxia and positive pressure. Liquid ventilation (LV) is an alternative strategy causing less lung cell damage (Mantell et al, 2002). In collaboration with Dr. Mantell's group, we investigated whether Cdk5 was involved in cell death induced by either GV or LV. Lung tissues were obtained after GV or LV, fixed and sectioned as described in Materials and Methods. Cell death and Cdk5 protein expression in these lung tissues were analyzed. TUNEL-fluorescence positive cells, i.e. the dying cells, in GV lungs were predominantly located at levels of bronchiole, either at the epithelial lining or clustered together in aggregates within the lumen (Fig. 3A). In contrast, TUNEL-positive cells are diffusely distributed throughout the parenchyma of LV lungs. No clusters of fluorescent signals were found at the more proximal airways of LV lungs as in GV lungs (Fig. 3B). Immunohistochemistry using anti-Cdk5 antibody showed that the patterns of Cdk5-positive cells in both GV (Fig. 3C) and LV (Fig. 3D) lungs were similar to that of TUNEL-positive cells, indicating a correlation of Cdk5 expression and cell death induced by ventilation in preterm lamb lungs. The data from this section in collaboration with Dr. Mantell have been published (Mantell et al, 2002).

D. Analysis of association of Cdk5 with cell death in cyclophosphamide-treated mouse embryo

a. Association of Cdk5 with cell death

Cyclophosphamide (CP), an alkylating agent that is commonly used in the treatment of cancers and progressive immune diseases, induces apoptosis mainly by causing DNA interstrand crosslinks. In mouse embryos, cell death is a prominent developmental event but its usual amount might not be enough for our study on cell death. Therefore, our lab has used CP to induce massive cell death in developing mouse embryos. Our lab has demonstrated an association between Cdk5 and apoptotic cell death in CP-treated mouse developing embryos (Zhu et al, 2002). Therefore, we hereby used CP-treated mouse embryo as a positive control model. Preparation of animals and tissues was as described in Materials and Methods. TUNEL staining revealed a large number of cells exhibiting fragmented DNA in the brain region of CP-treated embryo, indicating that these cells were undergoing apoptosis (Fig. 4A & B). Correspondingly, in the same brain region, immunohistochemistry using Cdk5 antibody showed many cells labeled brown, indicating Cdk5 was expressed in these cells (Fig. 4C & D). There was an undetectable level of Cdk5 protein in the same region of control embryos (data not shown). Comparison between the pattern of apoptotic cells and that of cells expressing Cdk5 in CP embryos further confirmed that Cdk5 is correlated with cell death.

The association between Cdk5 and cell death in CP embryos further supported that Cdk5 is associated with different pathological cell deaths as demonstrated in previous sections. The data from this section have been published (Zhu et al, 2002).

b. Uniqueness of correlation of Cdk5 with cell death among Cdks

As mentioned earlier in the Introduction, Cdk5 is a unique member of Cdk family in the sense that its functions identified so far unlike the other Cdks (Cdk1-4, 6-9) are not relevant to cell proliferation. Our lab has also shown that in the interdigital regions of developing mouse limbs, Cdk5 is the only Cdk whose protein expression corresponds to cell death (Ahuja et al, 1997). To determine whether Cdk5's uniqueness can be expanded to other systems, we examined the expression of several other members of the Cdk family, such as Cdk2, 3, 4, and 6, in CP-treated mouse embryos, by immunohistochemistry using specific antibodies. In accordance with their roles in cell cycle progression, none of them showed any correlation with cell death (Fig. 5A, B, D & E), unlike the strong immunoreactivity of Cdk5 in CP-treated embryos (Fig. 5C). Although Cdk2 was expressed in the treated embryos, its expression was only restricted to proliferating cells (Fig. 5A, white arrow) but not dying cells (Fig. 5A, black arrow). Therefore, we further concluded that the correlation with cell death is unique to Cdk5 among Cdk family members. The data of this section have been published (Zhu et al, 2002).

3. Discussion

In this study, we compared the pattern of Cdk5 expression and that of cell death in various induced pathology, including rat lungs with acute pneumonitis, *Leishmania major*-infected mouse limbs, preterm lamb lungs after liquid or gas ventilation, and CP-treated mouse embryos. As in other situations such as development, homeostatic maintenance and neurodegenerative pathologies, Cdk5 is correlated with cell death in various pathological situations. In addition, among different Cdks examined, only Cdk5 is associated with cell death in CP-treated mouse embryos. This corresponds to our previous finding in developing mouse limb (Ahuja et al, 1997). The generality and uniqueness of Cdk5 association with cell death in various systems, ranging from normal to induced cell death, in either developmental or adult systems, suggests a general Cdk5-involved signaling pathway during cell death. Furthermore, the fact that cell death is most likely to take different signaling pathways in these many different situations has raised the question of the interdependence between Cdk5 activation and other signaling pathways during cell death. We are going to discuss in more detail the regulation of Cdk5 activation and its relationship with other signaling pathways during cell death in following chapters.

Chapter II. Up-regulation of Cdk5 during cell death

A. Analysis of Cdk5 up-regulation during cell death at transcriptional level

B. Analysis of Cdk5 up-regulation during cell death at translational level

C. Analysis of Cdk5 up-regulation during cell death

at post-translational level

1. Objective:

In the previous chapter, we have demonstrated there is an up-regulation of Cdk5 protein during cell death as examined by immunohistochemistry. This increase of Cdk5 protein expression during cell death may result from an elevation of the level of Cdk5 transcript mRNA. There are many proteins whose up-regulation occurs at the transcriptional level during cell death. For example, the activity of Dronc, a key apical caspase in *Drosophila*, is transcriptionally up-regulated by ecdysonc in ecdysonc-mediated cell death during metamorphosis (Cakouros et al, 2002). In addition, increased protein translation of the available Cdk5 transcript may also result in an increased level of Cdk5 protein expression during cell death. Several cell death-related proteins are up-regulated at the translational level during cell death. For instance, the expression of Par-4 (prostate apoptosis response-4), a death domain-containing protein that plays an important role in neuronal apoptosis, is regulated at the tranlational level within synaptic compartments in neuronal cell death (Duan et al, 1999). Therefore, we evaluated the regulation of Cdk5 at both mRNA and protein synthesis levels during cell death using CP-treated mouse embryos as a model.

Furthermore, as a kinase, the activation of Cdk5 requires association with its regulatory proteins, among which p35 and p39-mediated Cdk5 kinase activity is essential and sufficient for neurodevelopment (Ohshima et al, 1996; 1999; Chae et al, 1997; Ko et al, 2001). But these studies were conducted only on Cdk5, p35, p39 and p35/p39 mutant mice, and the Cdk5 kinase activity was not actually examined. We therefore determined whether Cdk5 is activated at the post-translational level, i.e. kinase activity level, during cell death, using histone H1 as the *in vitro* substrate.

In this chapter, by examining the levels of Cdk5 messages, protein and kinase activity during cell death, we attempted to determine at which level(s) of transcriptional, translational and post-translational levels Cdk5 is up-regulated during cell death. The data from this chapter have been published (Zhu et al, 2002; Zhu, 2002).

2. Results:

A. Analysis of Cdk5 up-regulation during cell death at transcriptional level

The up-regulation of Cdk5 protein expression detected by immunohistochemistry in CP embryos (Chapter I) can result from an increased level of Cdk5 mRNA. We therefore examined and compared the levels of Cdk5 messages in control and CP embryos. For this analysis we used both northern blotting and *in situ* hybridization. Total RNA was extracted from control and CP embryos. The level of Cdk5 mRNA was detected via northern blot using a ^{32}P-labeled RNA probe. We found no change in message level between control (Fig. 6A, left lane) and treated embryos (Fig. 6A, right lane). This result was confirmed by *in situ* hybridization where sections of both control and CP embryos were hybridized with ^{35}S-labeled RNA probe. We did not see any difference in the level of mRNA expression in both control (Fig. 6B, a) and CP-treated mouse embryos (Fig. 6B, b). This result correlates with our earlier findings during normal limb development (Ahuja et al, 1997) and suggests that Cdk5 message is not up-regulated during cell death. This work was done in collaboration with Yong Zhu and has been published as part of his thesis "The study of the role of cyclin-dependent kinase 5 (Cdk5) during cell death" (Zhu, 2002).

Therefore, Cdk5 is not up-regulated at transcriptional level during cell death.

B. Analysis of Cdk5 up-regulation during cell death at translational level

Increased level of protein synthesis can result in up-regulation of Cdk5 protein expression during cell death as detected by immunohistochemistry. We therefore examined and compared the levels of *de novo* protein synthesis of Cdk5 in control and

CP mouse embryos. CP-treated embryos as well as control embryos were incubated in media containing ^{35}S-methionine and cysteine for 4 hrs to label synthesized proteins *in vitro*. The viability of the embryos was evaluated by embryonic heart beat. After the 4 hr labeling period, total proteins from both control and CP embryos were obtained using RIPA buffer as described in Materials and Methods. The whole protein lysates of both embryos were run on SDS-PAGE and separated proteins were transferred onto a nitrocellulose membrane. The labeled proteins were detected by autoradiograph, which showed many bands (Fig. 7A), indicating that many proteins were synthesized and labeled during the 4 hr incubation period. By western blot analysis using Cdk5 antibody, we detected Cdk5 protein in lysates of both control and CP embryos (Fig. 7B). To see if the Cdk5 protein in the whole lysate samples was also labeled, hence synthesized *de novo*, we performed immunoprecipitation using Cdk5 antibody. The labeled Cdk5 protein in the Cdk5 immunoprecipitates was evaluated by autoradiograph but did not give any band, indicating that there was no *de novo* synthesis of Cdk5 during the 4 hr period of CP-induced cell death (Fig. 7C). However, western blot using Cdk5 antibody showed the presence of Cdk5 protein in the Cdk5 immunoprecipitates of control and CP-treated embryos (Fig. 7D). This finding suggests that regulation of Cdk5 during cell death is most likely not translational. To rule out the possibility that the lack of Cdk5 ^{35}S-labeling in CP embryos is due to the limitation of *in vivo* embryo labeling, in which there might not be enough proteins being labeled for the short period of time (4 hr), my colleague Zhu examined Cdk5 ^{35}S-labeling during cell death induced by CHX in COS-7 cells for 18 hrs. Although Cdk5 protein was found in the Cdk5 immunoprecipitates of both untreated and CHX-treated COS-7 cells, he did not detect any *de novo* Cdk5 synthesis by

autoradiograph, indicating that Cdk5 is not up-regulated at translational level (Zhu, 2002). Therefore, the up-regulation of Cdk5 is likely to be post-translational modification. With this experiment we cannot rule out alteration in stability although this is unlikely since this should be reflected by western blot as well.

C. Analysis of Cdk5 up-regulation during cell death at post-transcriptional level

As a kinase, Cdk5 functions by phosphorylating its substrates. Since there is no up-regulation at either transcriptional or translational level, to address the possibility of post-translational modification, we examined the kinase activity of Cdk5 in both control and CP-treated embryos. After immunoprecipitation of equal amounts of lysates from control and CP embryos using Cdk5 antibody, Cdk5 kinase activity was assessed in an *in vitro* assay by detecting the incorporation of ^{32}P using histone H1 as a substrate. We found little or no activity in the control embryos (Fig. 8A, left lane). However, the CP treated embryos gave substantial kinase activity (Fig. 8A, right lane). Quantification via densitometry on kinase activities demonstrated a 3-fold increase in CP-treated mouse embryos compared to control ones (Fig. 8B). The detection of the kinase activity is indicative of activation of Cdk5 during cell death and therefore indicates that Cdk5 is up-regulated at the post-translational (i.e. kinase) level during cell death. The up-regulation of Cdk5 kinase activity has also been observed in COS-7 cells treated with either cycloheximide or camptothecin. This work was done in collaboration with Dr. Zhu and has been published (Zhu et al, 2002) and is also a part of Zhu's thesis "The study of the role of cyclin-dependent kinase 5 (Cdk5) during cell death" (Zhu, 2002).

3. Discussion

In this study, we have shown that Cdk5 is not up-regulated at either transcriptional or translational level, but at the level of post-translational kinase activity during cell death, in CP-treated mouse embryos. This same finding was also obtained in COS-7 cells treated with CHX (Zhu, 2002).

Previously, our lab has demonstrated that in developing mouse limb and brain, Cdk5 kinase activity is up-regulated in correlation with cell death (Ahuja et al, 1997). Since the increase of this kinase activity may result from the increase at one or more of the following levels: transcriptional, translational or post-translational, we started to determine the change at each level during cell death.

In this chapter, neither *in situ* hybridization nor northern blot showed any change in Cdk5 mRNA level of CP-treated mouse embryos compared to control embryos. Although there was a marked increase in Cdk5 protein expression detected by immunohistochemistry in CP-treated embryos, western blot did not find any significant change in Cdk5 protein levels between control and treated embryos. The abundant Cdk5 protein detected by immunohistochemistry could reflect a structural rearrangement that enables better antibody recognition in this situation. This can be due to a modification of the protein such as phosphorylation or alteration of its localization within the cell during cell death.

Studies have shown that p25, the truncated p35, mediates Cdk5 to localize to cytosol during cell death, compared to a normal membrane-bound localization when associated with p35 in normal situations (Partick et al, 1999). ^{35}S-labeling of *de novo*

synthesized proteins indicated that Cdk5 is a very stable protein and is not newly made in response to cell death induction. This result is consistent with Zhu's finding that Cdk5 is also not labeled during cell death in COS-7 cells (Zhu, 2002), further indicating there is no up-regulation of *de novo* Cdk5 protein synthesis during cell death. In CP-treated embryos, Cdk5 activity was 3 times that of control embryos. Therefore, during CP-induced embryonic cell death, the up-regulation of Cdk5 appears to be at the kinase level.

We postulate that Cdk5 is up-regulated at its kinase level during cell death, and Cdk5 activity plays a key role in cell death. This has been more and more verified by the recent findings of the involvement of Cdk5 activation in neuronal cell death (Lee et al, 2002; Nath et al, 2000; Fu et al, 2002; Hashiguchi et al, 2002; Patzke & Tsai, 2002). However, how Cdk5 activation is regulated during cell death and how this activation fits with other cell death signaling pathways have yet to be explained; these questions are the main topics of the following chapters.

Chapter III. Dependency of regulation of Cdk5 activation on p53 function during cell death

A. Analysis of cell death induced by different stimuli in p53+/+ and p53-/- cells

B. Analysis of expression and activation of Cdk5 during cell death in p53+/+ and p53-/- systems

C. Analysis of regulation of Cdk5 activation during cell death in p53+/+ and p53-/- cells

D. Analysis of kinetics of cell death induction in p53+/+ and p53-/- cells

1. Objective:

In previous chapters (Chapter I, II), we have shown that Cdk5, a unique member of Cdk family, is activated during cell death in different systems, indicating a general Cdk5-involved cell death signaling pathway. As illustrated earlier in the Introduction, the p53 signaling pathway, a well-known pathway, plays a crucial role in the execution of apoptosis (Lowe et al., 1993; Clarke et al., 1993; for review, see Hickman et al, 2002). It has been shown that activation of p53 is essential for neuronal apoptosis during brain development (Aloyz et al, 1998). Cdk5 activity also plays an important role in both neuronal differentiation (Tsai et al, 1993; Nikolic et al, 1996; Fu et al, 2002) and neuronal cell death (Patrick et al, 1999; Lee et al, 2000; Nath et al, 2000; Town et al, 2002; Tseng et al, 2002). Taken together, it appears that the p53 activity and Cdk5 activity that are required during neuron development, may also have some interaction during cell death. Since Cdk5 is also involved in non-neuronal cell death as so is p53, the interaction of them may also be seen in non-neuronal situation as well. This is the question we would address in this chapter.

During cell death, the induction of many p53 target genes can be p53-dependent, such as Bid, Puma and Noxa, which were already mentioned in the Introduction and are induced in mouse jejunum and ileum during gamma-irradiation-induced cell death (Fei et al, 2002). On the other hand, the activation of many other cell death-related genes during cell death can be p53-independent. For example, both initiator caspases-2, -8 and –10 and effector caspases-3, -6 and –7 are activated in the absence of p53 in dying Ewing's sarcoma family tumor cells (ESFT) treated with basic fibroblast growth factor (bFGF, Westwood et al, 2002). We therefore asked whether Cdk5 activation is p53-dependent or

-independent during cell death. Furthermore, as mentioned in the Introduction, studies including ours have shown that Cdk5/p25 activation is correlated with cell death involved in neurodegeneration (Patrick et al, 1999; Lee et al, 2000; Town et al, 2002; Tseng et al, 2002), neuronal injury by ischemia (Nath et al, 2000), teratogen-treated developing mouse embryos (Zhu et al, 2002), and chemical compound-treated COS-7 cells (Zhu, 2002). It has been further demonstrated that the induction of p25 and the subsequent activation of Cdk5 result from calpain activation during neuronal cell death (Patrick et al, 1999; Lee et al, 2000; Nath et al, 2001). Therefore, we also evaluated the dependency of calpain and p25-mediated Cdk5 activation on p53 during cell death.

Taken together, in this section, we used both p53-/- fibroblast cells and p53-deficient mouse embryos as model systems, and examined Cdk5 activation and its regulation during cell death with regard to p53 function.

2. Results:

A. Analysis of cell death induced by different stimuli in p53+/+ and p53-/- cells

a. Cell death induction in C8 and A9

To establish a model system in which we could examine the dependency of Cdk5 activation during cell death on p53, we first had to establish a p53-independent cell death model. We started with C8 and A9 cells, which are wild type and p53-/- mouse embryonic fibroblast cells oncogenically transformed with both E1A and ras, respectively (Materials and Methods of Lowe et al, 1993). Insults such as ethanol (EtOH), cycloheximide (CHX) and camptothecin (CPT), can induce cell death in a variety of cells by interfering with different aspects of a cell's housekeeping activities. EtOH can damage cell membrane resulting in loss of membrane integrity (Rubin & Rottenberg, 1982). CHX is an inhibitor of protein synthesis (Gong et al, 1993). CPT hampers the function of DNA topoisomerase I (Morris et al, 2001). We therefore examined whether they could also induce cell death in C8 (p53+/+) and A9 (p53-/-) cells.

Both C8 and A9 cells were treated with EtOH (2.5%), CHX (25 μg/ml) or CPT (20 μM) for 18 hrs. Levels of cell death were determined by percentage of cells that were stained blue using trypan blue exclusion assay (Materials and Methods). As shown in Figure 9, both cells showed significant levels of cell death when treated with CHX or CPT after 18 hrs. The total cell death in C8 and A9 treated with CHX was 90.3% and 74.8%, respectively. CPT killed 100% of C8 and 57.7% of A9 cells. EtOH caused 77.2% cell death in C8 compared to a 17.5% cell death in A9 cells. However, untreated control C8 cells also showed 23.5% cell death after 18 hrs incubation, suggesting that C8 cells were very sensitive to lower serum concentration (1% FBS during treatment),

whereas untreated control A9 cells were unaffected by the low serum incubation with only 5.9% cell death after 18 hrs. We therefore corrected the data by subtracting the basal levels of cell death in untreated control C8 and A9 cells. By these calculations, CHX killed 66.8% and 68.9% of C8 and A9 cells, respectively. CPT induced 76.5% cell death in C8 compared to 51.8% killing in A9. EtOH killed 53.7% of C8 cells but did not induce significant (only 11.6%) cell death in A9, indicating a p53-dependent EtOH killing in C8. Although CHX and CPT could induced different levels of cell death in p53+/+ and p53-/- cells, they both caused statistically significant cell death. The cell death we found in A9 (p53-/-) cells treated with CHX and CPT suggested that this cell death is p53-independent. Comparing the levels of cell death induced by CHX or CPT in C8 and A9, cells deficient for p53 (A9) seemed to be less susceptible to CPT-induced cell death, but CHX did not cause different levels of cell death in C8 and A9 (Fig. 9).

Hence, we have now established a model in which both p53-dependent and –independent cell death can be induced, we are able to evaluate the nature of this cell death and the activation of Cdk5 during this cell death.

b. Apoptotic cell death induced in C8 and A9

All the three cell death inducers used above, EtOH, CHX and CPT, can induce either apoptosis or necrosis in different cell lines or in the same cell line under different conditions (Blom et al, 1999; Morris et al, 2001; Dalton SR et al, 2003). In order to evaluate the nature of cell death induced in C8 and A9, we checked whether the induced cell death in C8 and A9 was apoptotic. There are several hallmarks characterizing apoptotic cell death, among which fragmented chromatin and internucleosomal DNA

fragmentation are the most often used characteristics. To examine fragmented DNA of oligonucleosomal lengths, low molecular weight DNA was isolated from C8 and A9 cells after the cells were treated with EtOH, CHX or CPT for 18 hrs. Agarose gel electrophoresis of DNA samples from treated C8 and A9 showed typical DNA ladders representing cleaved DNA fragments of multiples of 180bp, which is the size of nucleosome (Fig. 10, lane 2, 3, 4, 6, and 8). Since EtOH induced only 11.6% cell death in A9 cells, there was barely detectable DNA ladder (Fig. 10, lane 7). There was a little detectable level of DNA ladder in untreated C8 control cells due to their high sensitivity to lower serum media as discussed before (Fig. 10, lane 1). There was no detectable DNA ladder in untreated A9 control cells (Fig. 10, lane 5).

We further confirmed the apoptotic nature of induced cell death in C8 and A9 by *in situ* terminal deoxynucleotidyl transferase-mediated d-UTP nick end labeling (TUNEL), which represents a more sensitive assay to detect fragmented DNA in apoptotic cells. After C8 and A9 cells were treated with CPT (20 μM) for 18 hrs, the cells were plated onto a slide and fixed with 3% paraformaldehyde followed by examination of DNA fragmentation via TUNEL assay, as described in Materials and Methods. In both C8 and A9 cells treated with CPT, fragmented DNA labeled brown by TUNEL assay was detected (Fig. 11C & D). There was no positive signal in control cells treated with only DMSO (Fig. 11A & B). In addition, nuclear condensation and fragmentation as detected by Hoechst staining was also seen in both C8 and A9 treated with CPT (Fig 11E & F). Similar data were obtained when C8 and A9 were killed by EtOH or CHX at different levels (data not shown). Our data therefore showed that both

C8 and A9 cells could be induced to die by apoptosis upon EtOH, CHX or CPT treatment.

Due to the possibility of producing differential results by different cell death detection methods based on their specificity, percent cell death has been quantified by three different cell death assays, including trypan blue exclusion, TUNEL assay and Hoechst staining. All three methods gave comparable levels of cell death in our experiments, and we therefore only showed cell death levels measured by trypan blue exclusion.

Caspase-3 is a pivotal executioner caspase, whose activation is often used as a hallmark of apoptosis. The appearance of some of the classical apoptotic morphologies, such as DNA internucleosomal fragmentation, is attributed to caspase-3 activation (Leist & Jaattela, 2001). We therefore asked whether caspase-3 was activated during cell death in C8 and A9 cells. From both C8 and A9 cells treated with EtOH, CHX or CPT for 18 hrs, equal amounts of lysates were run on a 15% SDS-PAGE. The activation of caspase-3 was demonstrated by the appearance of the cleaved active form of procaspase-3 at 17 kDa via western blot analysis using anti-active caspase-3 antibody (Fig. 12A). The activation of caspase-3 was further confirmed by examining the cleavage of poly(ADP ribose) polymerase (PARP), a substrate of caspase-3. C8 and A9 protein samples were separated by 10% SDS-PAGE and analyzed via western blot using anti-PARP antibody. The appearance of a band at 85 kDa indicated that PARP was cleaved to its active form by caspase-3 when C8 and A9 cells were induced to die (Fig. 12B). The basal level of caspase-3 activation in untreated C8 cells (Fig. 12A & B) corresponded with the 23.5% cell death due to low serum incubation for the 18 hr treatment (Fig. 9). This observation

indicated that cell death in both C8 and A9 cells by EtOH, CHX, or CPT activates the caspase-3 pathway. Since both cells showed caspase-3 activation, our data also indicated that activation of caspase-3 does not require p53 function.

Upon completion of the above observation, we were confident that we have set up a model system (C8 and A9 cell lines), in which p53-independent apoptosis can be induced by both CHX and CPT. Although our data suggest that EtOH-induced cell death is p53-dependent, we still used EtOH to induce cell death in C8 cells and evaluated the associated Cdk5 activation as a control in some of the following experiments.

B. Analysis of expression and activation of Cdk5 during cell death in p53+/+ and p53-/- systems

a. Expression of Cdk5 protein during cell death in C8 (p53+/+) and A9 (p53-/- cells)

Having established that apoptotic cell death can be induced in both p53+/+ and p53-/- cell model system, we could now ask the relationship between cell death and Cdk5 expression as well as activity with regard to p53 regulation during cell death. Both C8 and A9 cells were treated with EtOH, CHX or CPT for 18 hrs. Western blot analysis of equal amounts of lysates from C8 and A9 cells showed similar levels of Cdk5 protein expressed in both untreated control and treated cells (Fig. 13A). This is consistent with what we have previously demonstrated in a number of other model systems (Ahuja et al, 1997; Gao et al, 1997; Zhang et al, 1997; Zhu et al, 2002) that the level of Cdk5 protein does not change, and it represents a stable protein that is not regulated at protein level during cell death. Our data also indicated that the level of Cdk5 protein expression is not affected by p53 status.

b. Expression and activation of Cdk5 during cell death in wild-type and p53-/- mouse embryos

The independency of Cdk5 protein expression on p53 during cell death was further confirmed using p53-/- developing mouse embryos. p53-deficient mice develop normally, and are highly susceptible to tumorigenesis leading to an average life span of only 5 months (for review, see Donehower, 2002). This suggests that there are no severe defects in death pathways in p53-/- developing mouse embryos. In developing mouse embryos, at different stages such as ED13.5, 14.5 and 15.5 (data for 14.5 and 15.5 not shown), TUNEL assay on sections of wild type and p53-/- embryos exhibited significant levels of cell death in the brain of both embryos (Fig. 13B, a & b). On an adjacent serial slide, immunohistochemistry using anti-active form of caspase-3 antibody also gave strong immunoreactivity in the same area of both embryos (Fig. 13B, c & d), suggesting caspase-3 is activated during cell death in both wild type and p53-/- embryos. Similarly, the Cdk5 immunoreactivity was detected using anti-Cdk5 antibody in this region of both wild type and p53 knockout mouse embryos (Fig. 13B, e & f). Our *in vivo* data have shown an up-regulation of detection of Cdk5 in developmental death in both wild-type and p53-/- mice, although there was no change in the level and pattern of Cdk5 expression between the embryos of the two genotypes (Fig. 13B, e & f). Since this increased detection of Cdk5 in dead cells is most likely due to the activated form of Cdk5, we could conclude that p53 pathway is not required for Cdk5 activation during cell death.

Therefore, the protein expression and activation of Cdk5 during cell death appears

not to require p53 function as shown by both *in vitro* and *in vivo* systems. To more specifically confirm this, we further evaluated the activation of Cdk5 kinase during cell death in C8 and A9 cells.

c. Activation of Cdk5 during cell death in C8 (p53+/+) and A9 (p53-/-) cells

Previously, we have demonstrated that Cdk5 is up-regulated at its kinase level in cell death (Ahuja et al, 1997; Zhu et al, 2002). We hereby examined the kinase activity of Cdk5 during the induced cell death in both C8 and A9 cells. This type of analysis is more difficult to conduct using *in vivo* model system due to low number of dying cells diluted by living cells. Cdk5 kinase activity was measured via histone H1 kinase assay using Cdk5 immunoprecipitates from untreated and treated C8 and A9 cells. When cell death was induced in C8 and A9 cells treated with EtOH, CHX, or CPT for 18 hrs, Cdk5 kinase was activated, shown by the incorporation of ^{32}P into histone H1 (Fig. 14A). The levels of Cdk5 kinase activity quantified by densitometry (Fig. 14B) corresponded with the levels of cell death induced by EtOH, CHX or CPT (Fig. 9). For example, the levels of cell death induced by CHX (~66%) and CPT (~77%) were higher than those induced by EtOH (54%) in C8 (Fig. 9); correspondingly, Cdk5 activity caused by CHX (110%) or CPT (90%) was higher than EtOH-induced Cdk5 activation (60%) (Fig. 14B). There was a slight detectable level of Cdk5 activity in untreated C8 cells (Fig. 14A & B), due to their susceptibility to death when incubated in low serum media for 18 hrs as discussed before (Fig. 9). In A9 cells treated with CHX and CPT, relatively high levels of cell death were induced (CHX, 68.9%; CPT, 51.8%), Cdk5 was significantly activated (CHX, 70%; CPT, 60%; Fig. 14A & B). Since EtOH did not induce significant cell death in A9

cells (11.6%), there was no detectable level of Cdk5 activity when A9 cells were treated with EtOH (Fig. 9; Fig. 14A & B).

Therefore, our data suggested that there is an activation of Cdk5 during apoptosis, which is not p53-required. These data further supported what we have shown in Chapters I and II, i.e. there is a correlation between Cdk5 kinase activation and cell death induction and further indicated that p53 is not required for this correlation.

C. Analysis of regulation of Cdk5 activation during cell death in p53+/+ and p53-/- cells

a. Induction of p25 during cell death in C8 and A9

Having determined that Cdk5 is activated during cell death in both C8 and A9 cells, we further evaluated how Cdk5 activation is regulated during cell death in C8 and A9. As we have mentioned earlier, Cdk5 is activated by different regulatory proteins, such as p35 and p39 during neuronal development (Tsai et al, 1994; Tang et al, 1995), and their truncated fragments p25 and p29 involved in neuronal cell death (Patrick et al, 1999; Lee et al, 2002; Nath et al, 2000; Fu et al, 2002; Hashiguchi et al, 2002; Patzke & Tsai, 2002). Our lab has also shown p25 production associated with Cdk5 kinase activation during cell death in CP-treated mouse embryos (Zhu et al, 2002) and in COS-7 cells treated with CHX or CPT (Zhu, 2002). We therefore evaluated levels of p25 induction and its relationship with Cdk5 activity during cell death in both C8 and A9 cells.

Protein samples were prepared as in previous experiments from C8 and A9 cells treated with EtOH, CHX or CPT for 18 hrs. Western blot showed the appearance of a

band at 25 kDa position when cell death was induced by different stimuli (EtOH, CHX or CPT) in both C8 and A9 (Fig. 15A). The intensity of the level of this 25 kDa band was given by quantifying three individual blots using densitometry (Fig. 15B), which corresponded to the level of Cdk5 activity as measured by the kinase assay using histone H1 as the *in vitro* substrate (Fig. 14A & B), as well as the level of cell death (Fig. 9) in different treatments. The level of EtOH-induced p25 production in C8 (~10%) was sufficiently lower than CHX (~100%) and CPT (~60%) (Fig. 15B), corresponding with the much lower Cdk5 kinase activity induced by EtOH (~60%) than triggered by CHX (~100%) and CPT (~90%) (Fig. 14B). EtOH barely induced any induction of p25 in A9 (Fig. 15B), coinciding with the undetectable Cdk5 kinase activity and a low level (11.6%) of cell death (Fig. 9; Fig. 14A & B).

To further confirm that the induction of p25 results from p35 cleavage, we modified the level of p35 in C8 and A9 cells via retroviral vector carrying either sense or anti-sense p35 cDNA, and checked its effect on p25 induction as well as cell death induction. Retroviral transfection of C8 and A9 with either sense or anti-sense p35 constructs gave more than 90% transfection efficiency, indicated by comparing cells that fluoresced green due to carrying EGFP-containing constructs (Fig. 16B & D; data for A9 not shown) with total cells viewed under phase contrast (Fig. 16A & C; data for A9 not shown). When C8 and A9 cells were treated with CPT for 18 hrs, cells transfected with anti-sense p35 showed less cell death than cells transfected only with empty vector (Fig. 17A & B, dot bar). The level of cell death induced by CPT in antisense p35-transfected C8 cells was 77% compared to 94% cell death in vector-transfected cells (Fig. 17A). In A9, anti-sense p35 transfection reduced CPT killing by 15% (72%-57%; Fig. 17B).

Accordingly, in both C8 and A9 cells induced to die by CPT for 18 hrs, transfection with sense p35 increased cell death levels compared to vector-transfected cells (Fig. 17A & B, brick bar). In C8, there was no detectable increase of cell death in sense p35-transfected cells, which might be due to the already high cell death level (94%) in vector-transfected cells (Fig. 17A). In A9, there was a 13% elevation of cell death in cells containing sense p35 constructs (Fig. 17B).

Meanwhile, protein samples were obtained from both vector-transfected and anti-sense or sense p35-transfected C8 and A9 cells treated with CPT for 18 hrs. Correspondingly, western blot of these protein samples showed a decreased level of p25 induction in anti-sense p35-transfected C8 and A9 cells compared with in vector-transfected cells in CPT treatment, indicating a blockage of p35 expression by its anti-sense cDNA (Fig. 17C & D). Western blot also demonstrated an increased level of p25 induction in sense p35-transfected C8 and A9 cells likewise, due to the overexpression of p35 by its sense cDNA (Fig. 17C & D).

Taken together, our data supported the conclusion that the induction of p25 resulting from p35 cleavage correlates with Cdk5 activation during cell death, which does not require p53 function.

b. Activation of calpain during cell death in C8 and A9

Calpain has been documented to cleave p35 to p25, leading to elevated Cdk5 activation in neuronal cell death (Patrick et al, 1999; Lee et al, 2000; Nath et al, 2000). It remains to be evaluated whether calpain activation results in p25 induction during non-neuronal cell death. Since CP-treated mouse embryo is a well-established system, in

which Cdk5 activation is correlated with cell death as demonstrated in Chapter I and II, we first confirmed that the induction of p25 in non-neuronal cell death is due to calpain activation in this system.

Our lab has demonstrated that there was an induction of p25 in CP embryos, but not in control embryos, indicating that p25 production is associated with Cdk5 kinase activation during cell death in CP embryos (Zhu et al, 2002). We therefore examined whether calpain is activated and leads to p25 induction in CP-treated mouse embryos. Calpain activation can be determined by the cleavage of its endogenous substrate spectrin to form the specific spectrin breakdown products (SBDPs) at 150 kDa and 145 kDa (Wang, 2000). Although this antibody can also recognize active caspase-3-cleaved products of spectrin, the caspase-3 specific SBDPs are at 150 kDa and 120 kDa (Wang, 2000), which can be differentiated from calpain SBDPs. Using anti-spectrin antibody, by western blot analysis of proteins from control and CP-treated mouse embryos, we saw a dual band at 150 kDa and 145 kDa in CP-treated but not the control embryos, implicating calpain activation in CP embryos (Fig. 18B). Therefore, our data indicated a correlation between calpain activation and p25 induction as well as Cdk5 activation during CP-induced cell death.

After establishing that p25 is induced by calpain activation during non-neuronal cell death, we further examined calpain activation and its correlation with p25 induction during cell death in CP embryos, as well as its dependency on p53 in C8 and A9 cells. We also evaluated p25 production upon inhibition of calpain activity by specific calpain inhibitors in C8 and A9 cells, to verify the correlation between calpain activation and p25 induction during cell death.

C8 and A9 cells were incubated with different cell death inducers, EtOH, CHX or CPT, for 18 hrs. Using anti-spectrin antibody, western blot analysis of C8 and A9 protein after cell death induction exhibited a dual band at 150 kDa and 145 kDa, implicating calpain activation, in all the different situations leading to cell death (Fig. 19A). Furthermore, the level of calpain activation (Fig. 19A) corresponded to that of p25 induction (Fig. 15). To verify that the production of p25 resulted from calpain activation during cell death in both C8 and A9, we used two specific calpain inhibitors, calpastatin peptide (CS) and PD150606 (PD), to block calpain activation. Calpastatin is an endogenous inhibitor of calpain, and PD150606 is a synthetic inhibitor. Both inhibitors have been widely used to block calpain activity in different situations (Eto et al, 1995; Wang et al, 1996). In both C8 and A9 cells treated with CPT for 18 hrs, addition of either CS or PD eliminated the appearance of p25, in correlation with a reduced level of cell death by 20%, compared to cells treated only with CPT (Fig. 19B & C). Upon addition of either CS or PD, the dual band indicative of calpain activation disappeared in CPT-treated C8 and A9 (Fig. 19D), further confirming that calpain activation was blocked by CS or PD. This blockage study has also confirmed that the spectrin antibody-detected dual band represents calpain activation but not caspase-3.

Taken together, these results suggested that there exists a correlation between calpain-mediated p25 induction and Cdk5 activation during cell death, which does not require p53 function.

D. Analysis of kinetics of cell death induction in p53+/+ and p53-/- cells

a. Kinetic study of cell death induced by CPT in C8 and A9 cells

To further confirm the p53-independent cell death and Cdk5 activation in C8 and A9, we first examined cell death induced in C8 and A9 at two times after treatment, 8 hr and 18 hr. As shown previously, by 18 hr, both CHX and CPT induced more than 60% cell death in both C8 and A9, and EtOH caused about 80% cell death in C8 but not in A9 (Fig. 9). At 8 hr after treatment, only C8 cells displayed significant levels of cell death, ranging from 69% EtOH killing to 96% CPT killing with less than 15% cells showing trypan blue positive staining in A9 cells (Fig. 20A). Comparing levels of cell death at 8 hr and 18 hr in A9 cells, the fact that more than 50% of A9 cells died in CHX and CPT treatment after 18 hrs suggested that lack of p53 does not prevent cell death but only delays the onset and progress of cell death. Among the three death inducers (EtOH, CHX and CPT), CPT induced sufficient and differential levels of cell death after 18 hrs in C8 *vs* A9 cells (Fig. 10). We therefore evaluated in more detail the kinetics of CPT-induced cell death, as well as activation of caspase-3, Cdk5 and calpain at different times (0, 3, 6, 8, 12, 15 and 18 hr) during the 18 hrs treatment in both C8 and A9. As shown in Figure 21B, by 6 hr, C8 cells started to die at a sufficient level, with trypan blue exclusion assay showing a 49% cell death, comparing with only 4% death in A9. From 6 hr to 8 hr, the level of cell death in C8 elevated to 97%, whereas only about 10% A9 cells died by 8 hr. However, A9 cells started to die from 20% by 12 hr to 40% by 15 hr (Fig. 20B). Activation of caspase-3 shown by western blot using anti-active caspase-3 antibody indicated that the induction of a caspase-3-dependent cell death has already started at 6 hr in C8 (Fig. 21B). Correspondingly, by 6 hr, there started to appear a strong induction of

p25 (Fig. 21C) indicative of Cdk5 activation, and dual SBDPs at 150 kDa and 145kDa (Fig. 21D) implicative of calpain activation in C8. On the other hand, in A9, one must wait until 12 hr to detect the appearance of an activation of caspase-3 (Fig. 21B), an induction of p25 (Fig. 21C), and activation of calpain implicated by the appearance of SBDPs at 150 kDa and 145 kDa (Fig. 21D). The level of Cdk5 protein during the 18 hr period stayed the same in both C8 and A9 (Fig. 21A, data for 8, 15 and 18 hr not shown).

These data suggested that there is a delayed induction of cell death in cells lacking p53, corresponding to a delayed activation of caspase-3, Cdk5 and calpain in CPT cell killing. The finding that caspase-3 is activated during CPT induced cell death in A9 cells, further indicated that CPT-induced cell death undergoes a p53-independent and caspase-3-activated pathway.

b. Kinetic study of cell death induced by ultraviolet (UV) in C8 and A9 cells

The above study on kinetics of CPT killing regarding p53 genotypes indicated that p53-/- cells are less susceptible to CPT induced cell death. This finding is consistent with studies that p53-deficient human fibroblast cells exhibit an enhanced resistance to ultraviolet (UV)-induced cell killing (Barley et al, 1998). Due to the fact that both CPT and UV are DNA-damaging agents, we examined the kinetics of UV killing in both C8 and A9 cells to further confirm the delayed onset of cell death in p53-deficient cells and the correspondingly delayed activation of Cdk5.

Both C8 and A9 cells were irradiated by 254nm UV (50 J/m^2) and cultured in 1% serum media afterwards for 18 hrs. At four time points (0, 8, 12, and 18 hr), cell death levels were measured by trypan blue assay, and protein samples were extracted from C8

and A9 cell lysates for western blot analysis. As shown in Figure 22, cell death in C8 cells reached significance by 8 hr after UV irradiation, with 35.2% cells stained blue by trypan blue dye. By 12 hr, cell death climbed to 75.4%, and by the end of this experiment, 91.8% C8 cells were dead (Fig. 22). In contrast, A9 cell did not start to die until after 12 hr with 32.7% of the cells displaying blue staining. Furthermore, A9 cells died at a much slower pace than C8, with only 58.4% cell killing by the end of 18 hr (Fig. 22). Our finding that A9 cells treated with 254 nm UV (50 J/m^2) displayed slower death kinetics than C8 Cells, was further confirmed by the delayed activation of caspase-3 during UV killing. Western blot of protein samples from UV-treated C8 and A9 cells using active caspase-3 antibody exhibited an activation of caspase-3 at as early as 8 hr in C8, and only after 12 hr in A9 (Fig. 23B). Correspondingly, the induction of p25 band began at 8 hr in C8, compared with a later appearance at 12 hr in A9, indicating a delayed Cdk5 activation (Fig. 23C). As always, Cdk5 protein level stayed the same during this 18 hr UV treatment, shown by its constant level on western blot (Fig. 23A). Therefore, as in CPT killing, A9 cells treated with UV due to lack of p53 showed a significantly decreased susceptibility to cell death than C8 cells and exert a correspondingly delayed activation of Cdk5.

Taken together the data obtained from the kinetic studies on both CPT and UV killing in C8 and A9, lack of p53 delays cell death induction, corresponding to a delayed activation of caspase-3, Cdk5 and calpain. This might be due to the fact that p53 is a pro-apoptotic factor, and cells lacking p53 have to find different pathways to die when cell death is induced. Hence, p53 seems to lower the threshold of the entry and commitment to cell death.

3. Discussion

In our study, cell death inducers, such as CHX, CPT and UV, induced significant levels of cell death in C8 and A9 cells after 18 hrs, suggesting that CHX, CPT, or UV-mediated cell killing is p53-independent. In this p53-independent cell death, we found that Cdk5/p25 activation is mediated by calpain activation. When we further examined the kinetics of cell death induced by either CPT or UV, there was a delay in the induction and progression of cell death in p53-/- A9 cells. During the progress of cell death in both C8 and A9 cells, the level of cell death correlated with activation of Cdk5 indicated by the induction of p25, and activation of calpain. Therefore, our kinetic study further confirms the hypothesis that p53 is not required for the calpain-mediated Cdk5/p25 activation that is associated with the induction of cell death. Furthermore, the finding that EtOH killed only less than 11.6% of A9 cells after 18 hrs compared to a 53.7% killing of C8 indicates a p53-dependent EtOH cell killing in these fibroblasts. However, the 11.6% of A9 cells that were eventually killed by EtOH died through a p53-independent pathway since A9 cells are p53-deficient. These findings suggest that different cell death inducers have different p53 dependency and elicit different pathways leading to cell death. However, no matter whether the cell death is p53-dependent or not, Cdk5/p25 activation associates with the induction of cell death, and this activation and generation of p25 are coincident with the activation of calpain. Furthermore, blockage of calpain using the two calpain inhibitors, CS or PD, prevented activation of Cdk5, and reduced the level of cell death by 20%. Therefore, there appears to be a direct correlation between the induction of cell death and activation of Cdk5/p25 in this system. This

confirms that calpain-mediated Cdk5/p25 activation also plays an important role in non-neuronal cell death. The consistent level of Cdk5 protein in C8 and A9 cells either untreated or treated with different stimuli and at different times during the kinetic study supports the argument that Cdk5 is a relatively stable protein and its activation during cell death is not regulated at the protein level, as we have previously stated (Zhu et al, 2002).

The fact that we only had 20% but not 100% cell death recovery when calpain-mediated Cdk5/p25 activity was blocked by calpain inhibitors suggests a Cdk5-independent cell death pathway; alternatively p25 may not be the only activator of Cdk5 during cell death, further indicating that cell death can take different signaling pathways, eventually leading to cell death through one way or the other after cell death is committed. One example of the existence of the Cdk5-independent pathway is the observation that neurons from Cdk5 knockout mice can die via apoptosis (Li et al, 2002).

Recently, Zhang and colleagues found that in apoptotic differentiated PC12 cells p53 level increases concomitantly with levels of Cdk5, and increased Cdk5 activity significantly elevates p53 transcriptional activity, and message and protein levels of p53 as well as the p53-responsive genes, such as p21 and Bax (Zhang et al, 2002). Synthetic p53 has been shown to be subject to Cdk5 phosphorylation (Zhang et al, 2002). Furthermore based on our finding that Cdk5 activation during cell death is p53-independent, but cells containing p53 gene are more sensitive to death, we hypothesize that Cdk5 kinase can work through p53 by phosphorylating p53 to trigger downstream events when cell death is induced. We are now working to see whether p53 is a direct substrate of Cdk5 during cell death in our systems, such as C8 cells. In the experiment, we will treat C8 cells with CPT with or without the calpain inhibitors as used before in

the presence of $^{32\gamma}$P-ATP for 18 hrs, since CPT induces significant levels of Cdk5 activation in correlation with cell death and calpain inhibition abolishes this activation of Cdk5. The labeled p53 will be detected by autoradiograph of the p53 immunoprecipitates from different C8 lysates. We will then compare the levels of phosphate-labeled p53 between when Cdk5 is activated and when Cdk5 activation is inhibited by calpain inhibitors in C8 cells treated with CPT. If the level of p53 phosphorylation is significantly higher when Cdk5 is activated, we will be able to conclude that p53 is a substrate of Cdk5 during cell death. This will shed light in more detail on the interdependency between Cdk5 and p53 pathways during cell death.

Developing brain of wild-type and p53-/- mice also exhibited an activation of caspase-3 during developmental cell death, indicating a p53-independent but caspase-3-mediated cell death involved in mouse brain development. This confirmed the important role of caspase-3 activation during development, which has been demonstrated by the fact that mice lacking caspase-3 have massive brain deformities and die embryonically (Kuida et al, 1996). This importance of caspase-3 activity in development further led us to study the dependency of Cdk5 activation on caspase-3 activation during cell death, which will be discussed in Chapter IV.

Chapter IV. Dependency of Cdk5 activation on caspase activity during cell death

A. Dependency of Cdk5 activation on Apaf-1 and caspase-9 activity during cell death

B. Dependency of Cdk5 activation on caspase-3 activity during cell death

C. Dependency of Cdk5 activation on caspase cascade during cell death

1. Objective:

As discussed in the Introduction, proteases are important components of cell death machinery, and proteolytic cleavages play a key role in cell death. Caspases are among the most important and most widely studied proteases involved in cell death. Although different signaling pathways are achieved in response to different stimuli, they always converge to a common mechanism leading to the cell's demise, by activation of caspases. Activation of caspases mediates two major cell death pathways: extrinsic and intrinsic. In the extrinsic pathway, the association of death factors to their ligands initiates activation of caspase-8, followed by caspase-3, -6 or –7 activation (Juo et al, 1998). Formation of Apaf-1/cytochrome c apoptosome, and activation of caspase-9 and –3, -6 or –7 are involved in the intrinsic pathway (Slee et al, 1999; Zou et al, 1999; Jiang et al, 2003).

As discussed in the Introduction, the defects displayed in different caspase knockout mouse models indicate that many major caspases, including caspases-3, 9 and caspase-activating factor Apaf-1, play an essential role in neuronal development (Colussi & Kumar, 1999). Cdk5 activation has also been shown to play a key part in neuronal development (Tsai et al, 1993; Nikolic et al, 1996; Fu et al, 2002) and neuronal cell death (Patrick et al, 1999; Lee et al, 2000; Nath et al, 2000; Town et al, 2002; Tseng et al, 2002). However, the relationship between Cdk5 activation and caspase cascade during cell death has yet to be revealed.

In this chapter, we examine whether activation of caspases is required for Cdk5 activation during cell death. To evaluate the dependency of Cdk5 on different caspases, such as caspase-3, -9, and Apaf-1, due to their important roles in the caspase cascade, we

used caspase-3- and Apaf-1-deficient cells and embryos, as well as caspase-9 null cells. Finally, to further confirm the independency of Cdk5 activation on the caspase cascade during cell death, we inhibited the activation of different caspases in C8 using zVAD and evaluated the activation of Cdk5.

2. Results:

A. Dependency of Cdk5 activation on Apaf-1 and caspase-9 function during cell death

Mitochondria-mediated cell death pathway is considered to be the intrinsic signaling pathway. In this process, cytochrome c is released upon mitochondrial permeablization transition (MPT) during apoptosis, and initiates dATP-dependent oligomerization of Apaf-1 leading to formation of a large multimeric Apaf-1/cytochrome c apoptosome. The apoptosome is sufficient to recruit and activate procaspase-9. Once activated, caspase-9 disassociates from the apoptosome and becomes available to cleave and activate downstream executioner caspases, such as caspase-3 (Zou et al, 1999). Since Apaf-1 and caspase-9 are two major players in the intrinsic caspase cascade, we examined whether Cdk5 activation during cell death is Apaf-1- and caspase-9-dependent or –independent. We used both *in vivo* developing mouse embryo and *in vitro* cell line model systems.

a. Dependency of Cdk5 activation on Apaf-1 function during cell death

As mentioned in the Introduction, the brain development in Apaf-1-deficient mice is profoundly affected, exhibiting a variety of hyperplasias and a lower incidence of apoptotic cells in their hindbrains compared to wild type mice. Apaf-1 knockout mice also exhibit delayed interdigital mesenchymal cell death and alterations in the development of lens and retina (Colussi and Kumar, 1999). We conducted TUNEL assay and immunohistochemistry to examine cell death and Cdk5 protein expression on developing Apaf-1 null mouse embryos of ED12.5, 13.5 and 15.5. Since the three

different developing stages gave similar level of cell death and Cdk5 protein expression, we showed only the results from ED13.5 embryos. In ED13.5 Apaf-1 knockout mouse embryos, many cells in the periphery of the liver were stained brown by TUNEL assay, indicating that these cells contained fragmented DNA and were undergoing apoptosis (Fig. 24A, black arrows). Immunohistochemistry using Cdk5 antibody exhibited many cells expressing Cdk5 protein in an adjacent section of the same region (Fig. 24B, black arrows). Furthermore, we saw an increased detection of Cdk5 protein expression due to the active form of Cdk5 in cells giving positive TUNEL labeling in both embryos (Fig. 24A & B). Therefore, Cdk5 appeared to be activated in dying cells in Apaf-1 knockout mouse embryos.

To more specifically evaluate whether Apaf-1 is required for Cdk5 activation during cell death, we used Apaf-1-/- cells. Primary mouse embryonic fibroblast cells lacking Apaf-1 and wild type cells were kind gift from Dr. Lowe (CSHL; Materials and Methods). Both cells were treated with CHX (25 μg/ml) or CPT (20 μM) for 45 hrs. By 24 hrs post-treatment, we examined the level of cell death, and did not see any obvious cell death in both Apaf-1-/- and wild-type cells. However, by 45 hrs after treatment, CHX induced 40% cell death in wild type cells and 17% death in Apaf-1-/- cells. CPT induced 45% death in wild type and only 20% cell death in Apaf-1-/- cells (Fig. 25A). This indicated that Apaf-1-deficient cells are more resistant to cell death, corresponding to that Apaf-1 is an apoptogenic factor. This is also consistent with the finding that Apaf-1 null fibroblast cells are relatively resistant to apoptotic insults, such as tamoxifen (Rao et al, 2002).

Protein samples were obtained from wild-type and Apaf-1-/- cells treated with CHX and CPT for 45 hrs. Western blot of equal amounts of protein samples from both wild-type and Apaf-1-/- cells using Cdk5 antibody showed there was no change in the level of Cdk5 protein in both cells before or after the induction of cell death (Fig. 25B). However, western blot of the same protein samples as above using p35 antibody exhibited the appearance of p25 induction in both cells induced to die by CHX and CPT (Fig. 25C). Since p25 induction is an indicator of the activation of Cdk5 during cell death as presented earlier (Zhu et al, 2002; Zhu, 2002), this also indicated that Cdk5 is activated in both wild-type and Apaf-1-/- cells induced to die by CHX and CPT. Additionally, the level of p25 induction, which correlates with the level of Cdk5 activity, paralleled to that of cell death (Fig. 25A & C). Taken together, our data showed that the activation of Cdk5 during cell death does not require Apaf-1.

b. Dependency of Cdk5 activation on caspase-9 activity during cell death

Deletion of caspase-9 in mice yields a phenotype essentially identical to that of Apaf-1 null mice, displaying profound defects in the developing brain (Hakem et al, 1998). Since the activation of caspase-9 depends on the functional cytochrome c/Apaf-1 apoptosome, the independency of Cdk5 activation on Apaf-1 function during cell death as demonstrated above may suggest that Cdk5 activation during cell death is also caspase-9-independent. To examine this hypothesis, we used caspase-9 knockout cells. Both wild type and caspase-9 -/- primary mouse embryonic fibroblast cells were kind gift from Dr. Lowe (CSHL; Materials and Methods). Both cells were treated with CHX (25 μg/ml) or CPT (20 μM) for 24 hrs and the level of cell death was determined by trypan

blue exclusion assay. 24 hrs after treatment, CHX induced 88% and 46% cell death in wild-type and caspase-9 knockout cells, respectively. CPT killed 65% wild-type and 50% caspase-9 -/- cells (Fig. 26A). Therefore, cells lacking caspase-9 seemed to be more resistant to cell death induction, which may be due to the fact that caspase-9 is a pro-apoptotic factor. This is also consistent with the finding that the dominant negative caspase 9 expressing ovarian cancer PA1 cells are resistant to chemotherapeutic agent killing (Wu & Ding, 2002). Protein samples were obtained from both cells after different treatments as mentioned in Materials and Methods. Western blot of these protein samples using Cdk5 antibody displayed a constant level of Cdk5 protein in both wild type and caspase-9 -/- cells with or without cell death induction (Fig. 26B). However, western blot using p35 antibody showed an induction of p25 when cell death was induced by either CHX or CPT in both cells (Fig. 26C), which is indicative of the activation of Cdk5 during cell death (Zhu et al, 2002; Zhu, 2002). Furthermore, the level of the p25 induction (Fig. 26C) corresponded with that of cell death measured by trypan blue exclusion (Fig. 26A) in both wild-type and caspase-9-/- cells. The above data suggested that Cdk5 is activated during cell death, which is caspase-9-independent.

Therefore, taken together, our data suggest that the activation of Cdk5 during cell death does not require Apaf-1-and caspase-9.

B. Dependency of Cdk5 activation on caspase-3 activity during cell death

The activation of caspase-3 is generally a downstream event in cell death signaling transduction, which can be achieved either by caspase 9 or by caspase 8. As discussed in the Introduction, caspase-3 plays an indispensable role in brain development,

and caspase-3 knockout mice die just before or upon birth exhibiting a variety of hyperplasias and disorganized cell deployment in the cerebral cortex, the hippocampus, and the striatum (Kuido et al, 1996). It has also been shown that Cdk5 is crucial in neurodevelopment. Mice lacking Cdk5 die perinatally, with significant defects of neurite outgrowth, neuronal migration, axonal guidance, and lamination (Ohshima et al, 1996). The fact that both caspase-3 pathway and Cdk5 activation play important roles in neuronal development prompted us to evaluate the interdependence of Cdk5 and caspase-3 activation during cell death. Since caspase-3 is downstream of caspase-9, the independency of Cdk5 activation on caspase-9 does not necessarily mean that Cdk5 activation is caspase-3-independent. We therefore asked whether Cdk5 activation during cell death is caspase-3-dependent or -independent.

We started by examining the relationship between Cdk5 activation and caspase-3 activity in caspase-3-deficient mouse embryos. Embryonic day (ED) 13.5 caspase-3 null mouse embryos were fixed and sectioned as described in Materials and Methods. TUNEL assay and immunohistochemistry were conducted on near serial sections. TUNEL assay showed cells with brown staining in the olfactory lobe of brain, indicating dead cells with fragmented DNA (Fig. 27A, black arrows). This suggested that some neurons die in a caspase-3-independent manner. Immunohistochemistry using Cdk5 antibody exhibited the expression of Cdk5 protein in many cells in the same brain area on an adjacent section (Fig. 27B, black arrows), indicating a caspase-3-independent Cdk5 expression in neurons. There seemed to be an increased detection of Cdk5 protein expression in dying cells (Fig. 27B, black arrows). This is due to the production of the active form of Cdk5 protein during cell death, which is more easily recognized by the

antibody as discussed in Chapter II. These data suggested that Cdk5 is activated during cell death in a caspase-3-independent manner.

To more specifically confirm that Cdk5 is activated during cell death under caspase-3 null condition, we used caspase-3 -/- primary mouse embryonic fibroblast cells. Both wild-type and caspase-3 null primary mouse embryonic fibroblast cells were gift from Dr. Lowe (CSHL; Materials and Methods). Two chemicals, CHX (25 μg/ml) and CPT (20 μM), were used to induce cell death in wild-type and caspase-3-/- cells. 24 hrs after treatment, trypan blue cell death assay showed 27% and 35% of caspase-3 -/- cells were killed by CHX and CPT, respectively, in contrast with an 87% cell killing by CHX and 63% by CPT in wild-type cells (Fig. 28). This finding is consistent with the pro-apoptotic property of caspase-3, and further suggested that cells lacking caspase-3 are less susceptible to cell death induced by CHX and CPT. However, the cell death that was induced in caspase-3-/- cells represented a caspase-3-independent mode of cell death.

Having established this model system in which caspase-3-independent cell death can be induced, we asked whether Cdk5 is expressed and activated in caspase-3 -/- cells during cell death induction. Cell lysates from both wild-type and caspase-3 knockout cells treated with either CHX or CPT for 24 hrs were equally loaded on SDS-PAGE and assayed by western blot analysis using Cdk5 antibody (Fig. 29A) and anti-p25 antibody (Fig. 29B). There was no detectable change in the level of Cdk5 protein between wild-type and caspase-3 -/- cells with or without cell death induction (Fig. 29A). However, p25 was induced in both wild-type and caspase-3 -/- cells induced to die by either CHX or CPT (Fig. 29B). To further verify that Cdk5 was activated during cell death induced in caspase-3 -/- cells, we performed Cdk5 kinase assay using histone H1 as an *in vitro*

substrate. The bands at 33 kDa given by phosphorylated histone H1 indicated activation of Cdk5 in both wild-type and caspase-3 -/- cells induced to die by CHX and CPT (Fig. 29C). The intensity of the phosphorylated histone H1 bands corresponded to that of p25 and induced cell death. This suggested that caspase-3 is not required for the p25-mediated Cdk5 activation during cell death.

C. Dependency of Cdk5 activation on caspase cascade during cell death

Data from the previous sections A and B suggest that Cdk5 can be activated during cell death in an Apaf-1-, caspase-9- and caspase-3-independent manner. Since Apaf-1, caspase-9 and –3 are the most important components of caspase cascade during cell death, these data also indicate that Cdk5 activation is likely to be caspase cascade-independent during cell death. We therefore evaluated the activation of Cdk5 during cell death when caspases are inhibited.

Previously, we have found cell death-associated Cdk5 activation in C8 cells, we therefore inhibited caspases activity in C8 cells to see whether Cdk5 activation was altered during cell death. C8 cells were treated with 20 μM of CPT in the presence or absence of 50 μM of zVAD-fmk for 18 hrs. zVAD-FMK (Z-Val-Ala-Asp(OMe)-CH_2F) is a highly specific, cell-permeable inhibitor of caspases, including caspases-1, -3, -4 and –7, and it prevents the processing of caspase-3 to its active form (17 kDa; Thornberry & Lazebnik, 1998). Trypan blue assay showed that zVAD protected C8 cells from CPT killing to some degree (Fig. 30). The level of cell death dropped from 97% when zVAD was not added to 71% in the presence of zVAD (Fig. 30). zVAD by itself did not cause cell death (17%) compared to control cells without either CPT or zVAD (18%) (Fig. 30).

Protein samples were obtained from C8 after different treatments and subjected to western blot analysis using different antibodies. Western blot analysis of the protein samples using active-caspase-3 antibody showed an induction of a 17-kDa band when C8 cells were treated with CPT only. This 17-kDa band represents the active form of caspase-3 resulting from cleavage of pro-caspase-3, indicating the activation of caspase-3 during cell death. On the other hand, the absence of this band suggested that caspase-3 activation was blocked by zVAD when C8 cells were coincubated with CPT and zVAD (Fig. 31B). Since caspase-3 is a downstream caspase that is activated by upstream caspases, such as caspase-9 or –8, inhibition of caspase-3 by zVAD indicated the blockage of different caspases.

After establishing that caspases were inhibited by zVAD in C8 treated with CPT, we examined the activation of Cdk5 in this cell death situation. Western blot analysis of the C8 proteins using p35 antibody showed an induction of p25 in C8 treated with CPT in the absence or presence of zVAD, indicating that Cdk5 is still activated even when different caspases were inhibited during cell death (Fig. 31C). Furthermore, the level of p25 induction, which represents the level of Cdk5 activation, corresponded to that of induced cell death. For instance, there was a decrease of p25 induction when C8 cells were treated with CPT and zVAD compared with CPT only treatment (Fig. 31C), which coincided with a 26% (97% CPT only; 71% CPT + zVAD) drop of cell death when zVAD was coincubated with CPT (Fig. 30). In the contrary, the level of Cdk5 protein expression shown by western blot stayed the same regardless of the different treatments (Fig. 30B).

These data supported the hypothesis that the caspase cascade is not required for Cdk5 activation during cell death.

3. Discussion

In this study, CHX and CPT both induced cell death in Apaf-1-/-, caspase-9-/- and caspase-3-/- cells, which represents an Apaf-1-independent, a caspase-9-independent and a caspase-3-independent cell death, respectively. In addition, CPT-induced cell death in Apaf-1-/-, caspase-9-/- and caspase-3-/- as well as in C8 when zVAD was added suggests a caspases-independent cell death. That Cdk5 is activated in these cell deaths, on one hand indicates that Cdk5 activation during cell death does not require Apaf-1-, caspase-9- and caspase-3 as well as the caspase cascade; and further supports the hypothesis of a general correlation between Cdk5 activation and cell death in different situations including different modes of cell death. Since caspases activity is necessary for the classical apoptotic morphologies such as internucleosomal DNA fragmentation (Leist & Jaatela, 2001), the Apaf-1-, caspase-9, caspase-3- and caspase cascade-independent cell death may represent a non-apoptotic mode of cell death. If so, we have demonstrated a correlation between Cdk5 activation and both apoptotic and non-apoptotic cell death. To more specifically evaluate the nature of these cell deaths, we are currently determining the internucleosomal DNA fragmentation, *in situ* DNA fragmentation and chromatin fragmentation in Apaf-1-/-, caspase-9-/-, and caspase-3-/- cells as well as C8 incubated with zVAD via agarose gel, TUNEL assay and Hoechst staining.

The independency of Cdk5 activation during cell death on caspases may also indicate that Cdk5 activation either is upstream of caspases activation or parallels to the caspase cascade. Sandal et al have found that Cdk5 activation is located upstream of caspase-3 activation and Cdk5 activity is needed for cleavage of pro-enzyme caspase-3 to

its active form (17 kDa), in cAMP-induced apoptosis in rat leukemia cells (Sandal et al, 2002). In addition, Cdk5-/- mice embryos develop normally until embryonic day (ED) 16.5, and reveal lesions and disruption in neuronal layering of many brain structures, including the cerebral cortex, hippocampus, cerebellum and olfactory bulb by ED18.5 (Oshima et al, 1996; 1999; Gilmore et al, 1998). However, by ED12, the brain development is already profoundly affected in caspase-3 null mice, displaying a variety of hyperplasias and a deployment of cell masses between the cerebral cortex, hippocampus and striatum due to loss of cell death (Kuida et al, 1996). The more severe defects at earlier developmental stages in caspase-3-/- mice indicate that Cdk5 activation may be upstream of or parallel to caspase-3 activation during normal neuronal development.

In contrast, Li et al's study has shown that there is caspase-3 activation during cell death in the brain of Cdk5 null mouse embryos (Li et al, 2002), which represents Cdk5-independent caspase-3 activation in neuronal cell death. Our finding has demonstrated that Cdk5 activation during cell death is caspase-3-independent. Therefore, these data together suggest that Cdk5 activation and caspase-3 activation may be independent of each other in neuronal cell death.

To evaluate whether Cdk5 activation is upstream of caspases and affects caspases activation or Cdk5 activation is irrelevant to caspases activation in our systems (such as C8), we will inhibit Cdk5 activation using either DN-Cdk5 or Cdk5 inhibitor roscovitine to see whether the activation of caspase-9 and –3 during cell death is altered or not. The finding will shed more light on the interdependence between Cdk5 activation and caspase cascade during cell death.

CONCLUSION

Conclusion

Previous findings from our lab and other groups have shown that Cdk5 activation is associated with apoptosis during development (Nikolic et al, 1996; Ahuja et al, 1997; Gao et al, 1997; Philpott et al, 1997; Pigino et al, 1997, 1998; Zhang et al, 1997; Musa et al, 1998, 2000; Session et al, 2001), in adult homeostatic maintenance of various tissues (Zhang et al, 1997; Lilja et al, 2001), and in neuronal cell death involved in neurodegeneration and neuronal injuries (Patrick et al, 1999, 2001; Lee et al, 2000; Nath et al, 2000; Bu et al, 2002; Hashiguchi et al, 2002; Kusadawa et al, 2002; Sisodiya et al, 2002). Furthermore, it has been shown that Cdk5 is activated by calpain-mediated p25 production during neuronal cell death (Lee et al, 2000; Nath et al, 2000).

In the study presented in this thesis, we first expanded the correlation between Cdk5 activation and cell death to a broader spectrum of systems, ranging from different pathological situations, including rat lungs suffering from *Streptococcus pneumoniae* type 25-caused pneumonia, mouse limbs infected with *Leismania major*, preterm lamb lungs after liquid or gas ventilation, and cyclophosphamide (CP)-treated mouse embryos, to different modes of cell death, including p53-, Apaf-1-, caspase-9, and caspase-3-independent cell death. Secondly, we further confirmed that Cdk5 is the only Cdk whose activation is associated with cell death, unlike other Cdks that are master regulators of cell cycle progression. Thirdly, we have demonstrated that Cdk5 is up-regulated at the post-translational level (kinase activity) during cell death. We next illustrated that Cdk5 activation during cell death is p53-independent, and apoptosome/caspase-9/caspase-3-independent, as well as likely to be caspase cascade-independent. Lastly, we have shown

that Cdk5 is activated by truncated p25 through calpain activation in non-neuronal cell death.

Having established that Cdk5 is activated in a variety of systems where cell death occurs, we can conclude that there is a general correlation between Cdk5 activation and cell death.

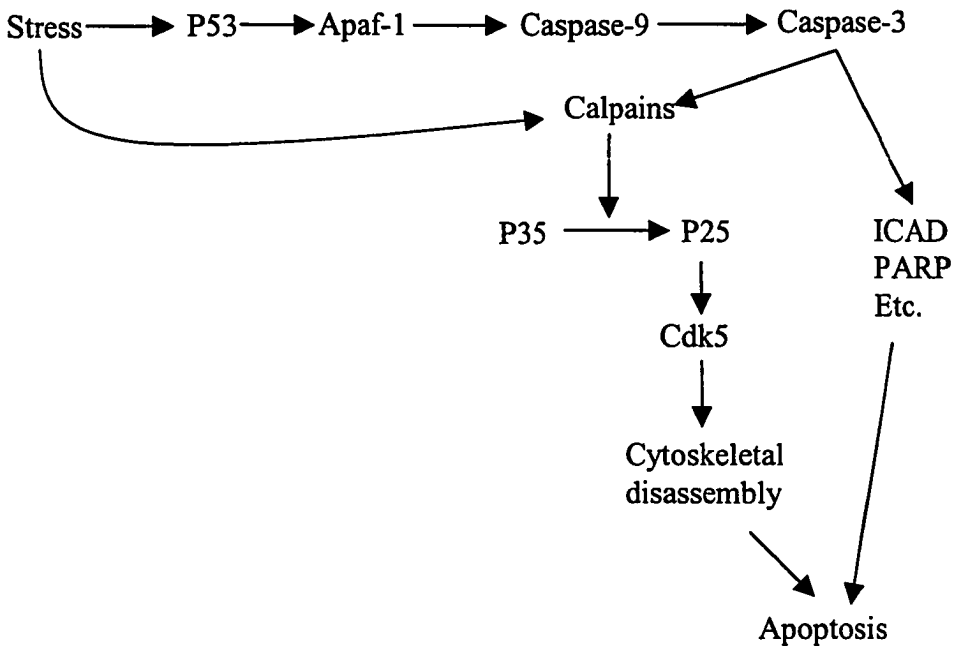
Given the fact that Cdk5 activation does not require p53, Apaf-1, caspase-9, -3 and possibly other caspases during cell death, it seems that Cdk5 activation overrides different cell death signaling pathways during cell death, and may be a downstream part of a general signaling pathway that leads to a variety of types of cell death.

In our study, we demonstrate that Cdk5 is activated by p25 and p25 results from p35 cleavage by calpain activation during non-neuronal cell death. Taken together with the Cdk5/p25 involvement in neuronal cell death shown by other groups, this finding suggests a general regulation of Cdk5 activity, which is calpain-mediated Cdk5/p25 activation in different types of cell death.

It has been shown that Cdk5/p35 can phosphorylate neurofilament proteins (Lee & Cleveland, 1996) and actin (Humbert et al, 2000a) during development. Furthermore, the microtubule-associated tau protein is the main substrate of Cdk5 kinase in pathogenesis of Alzheimer's disease (Alvarez et al, 1999). Therefore, we postulate a model of the correlation between Cdk5 activation and cell death: in various situations where cell death is triggered, either through p53-dependent or –independent, Apaf-1-dependent or not, caspases-dependent or –independent pathway, Cdk5 is activated through calpain-mediated p25 induction from p35 cleavage. Cdk5 activation causes the

phosphorylation of its substrates, possibly cytoskeletal components, leading to disassembly of the cellular cytoskeleton, which results in cell death (Diagram 2).

Diagram 2. The regulation of Cdk5 activation during cell death.



The above model is just one possibility about how Cdk5 activation mediates cell death. Since Cdk5 plays a key role in neuronal cell death, where reactive oxygen species (ROS) produced by the mitochondria is always involved (for review, see Fleury et al, 2002), we ask whether there is any interaction between Cdk5 activation and mitochondrial-mediated cell death pathway. In view of the general correlation between Cdk5 activation and cell death, we also ask whether calpain activation is the only upstream event that activates Cdk5 during cell death.

FIGURES AND FIGURE LEGENDS

Figure 1. Colocalization of TUNEL-positivity and Cdk5 expression in rat lung tissues at 4 d after *Streptococcus pneumoniae* type 25 infection.

S. pneumoniae type 25 (~5 x 10^6 cfu) were introduced into Wistar rats as described in Materials and Methods. Near serial sections were prepared.

A. TUNEL assay using fluorescein isothiocyanate (FITC)-conjugated antibody exhibited fragmented DNA in dying cells (green). 100X.

B. Cdk5 expression was detected with a Cy3-conjugated anti-Cdk5 antibody in cells expressing Cdk5 protein (red). 100X.

C. Composite using confocal microscopy showed the colocalization of Cdk5 expression and TUNEL positivity (yellow), indicating Cdk5 is expressed in dying cells. 100X

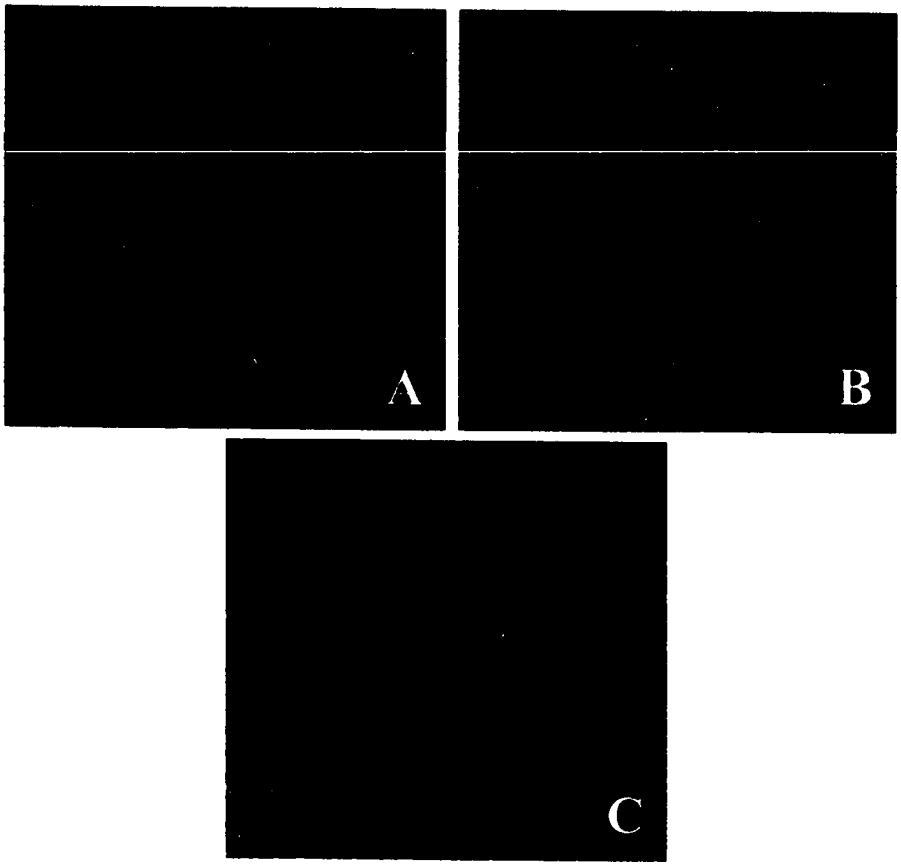


Figure 1

Figure 2. Cell death and Cdk5 expression in mouse draining lymph node after infection with 5 x 10^6 Lm promastigotes.

Near serial sections were prepared as described in Materials and Methods.

A & B. TUNEL assay using peroxidase-conjugated antibody and DAB as substrate, showed dying cells with fragmented DNA. A, 100X; B, 400X.

C & D. Cdk5 expression detected by immunohistochemistry using anti-Cdk5 antibody, displayed Cdk5-positive cells in the same area as in A& B on a near serial slide. C, 100X; D, 400X.

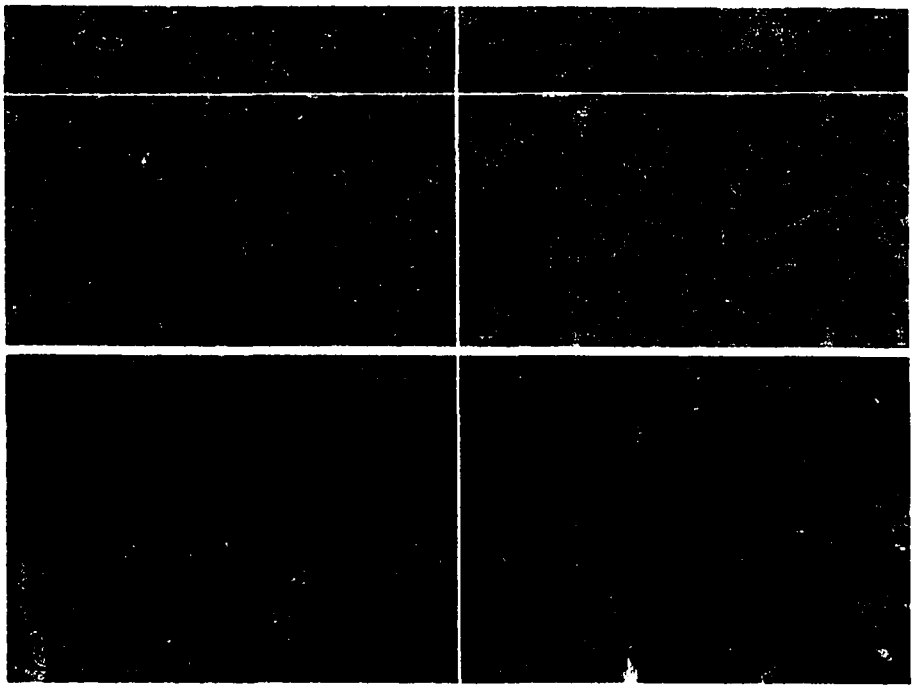


Figure 2

Figure 3. Cell death and Cdk5 expression in ventilated (GV and LV) lungs.

Preterm lambs of 120 gestation day received either GV or LV as described in Materials and Methods. Near serial sections were prepared.

A & B. Fluorescent TUNEL assay using FITC-conjugated antibody was performed on sections of lamb lungs ventilated for 4 d with either GV (A) or LV (B), exhibiting dying cells with fragmented DNA. Scale bars: 50 μm. 100X.

C & D. Cdk5 expression was characterized by immunohistochemical staining with anti-Cdk5 antibody in GV (C) and LV (D) lungs. Scale bars: 20 μm. 400X.

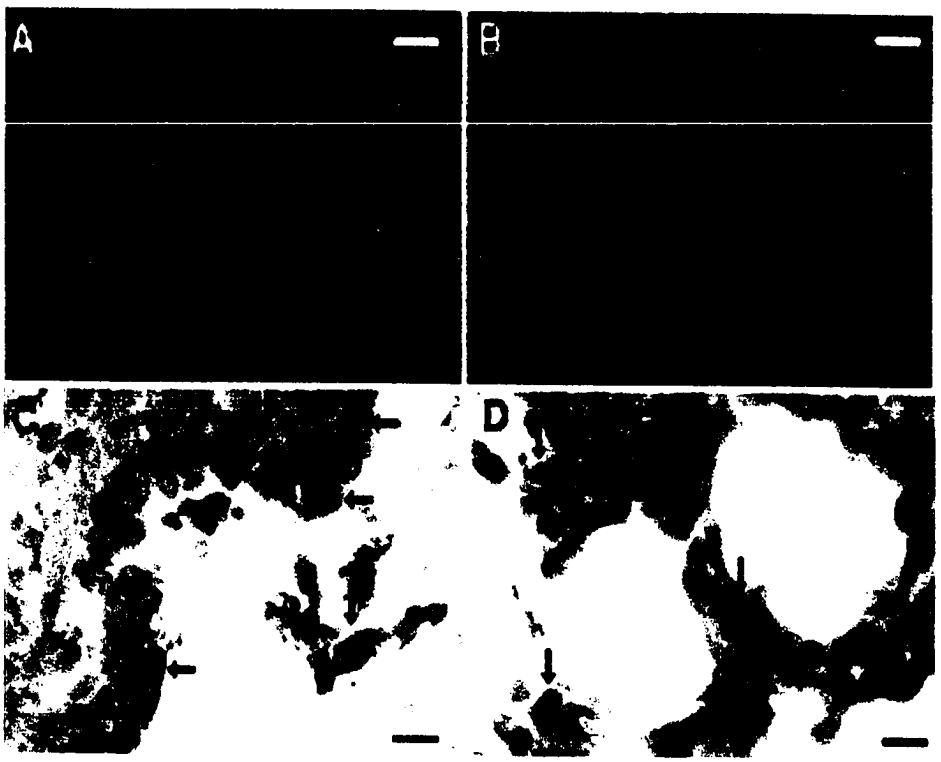


Figure 3

Figure 4. Cell death and Cdk5 expression in CP-treated mouse embryos by TUNEL assay and immunohistochemistry, respectively.

Pregnant female mice were injected with CP at gestation day 9.5, and embryos were removed 24 hrs later as described in Materials and Methods. Near serial sections were prepared.

A & B. DNA fragmentation by TUNEL assay showed many cells with fragmented DNA in CP embryos. A, 100X; B, 400X.

C & D. On the next serial section, many cells expressing Cdk5 protein were labeled by Cdk5 antibody. C, 100X; D, 400X.

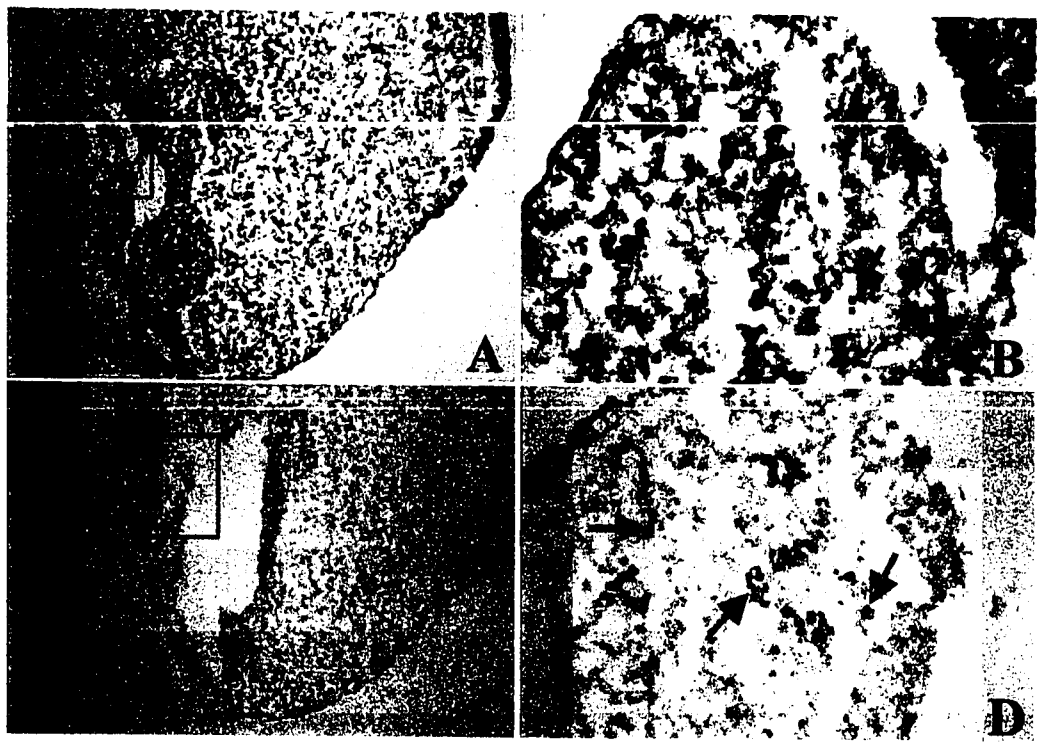


Figure 4

Figure 5. Cdk5 is the only Cdk whose expression is associated with cell death in CP-treated embryos.

The preparation was as in Figure 4. Near serial slides were exposed to different Cdk antibodies, Cdk2 (A), Cdk3 (B), Cdk4 (C), Cdk5 (D), and Cdk6 (E). While there were many cells labeled Cdk5 antibody, as indicated by the arrows in (D), there was no labeling by Cdk3, 4 and 6 antibodies (B, C, and E). Although Cdk2 antibody labeled some cells (A) this label was confined to proliferating cells (A, white arrows) but not dying cells (A, black arrows). 400X.

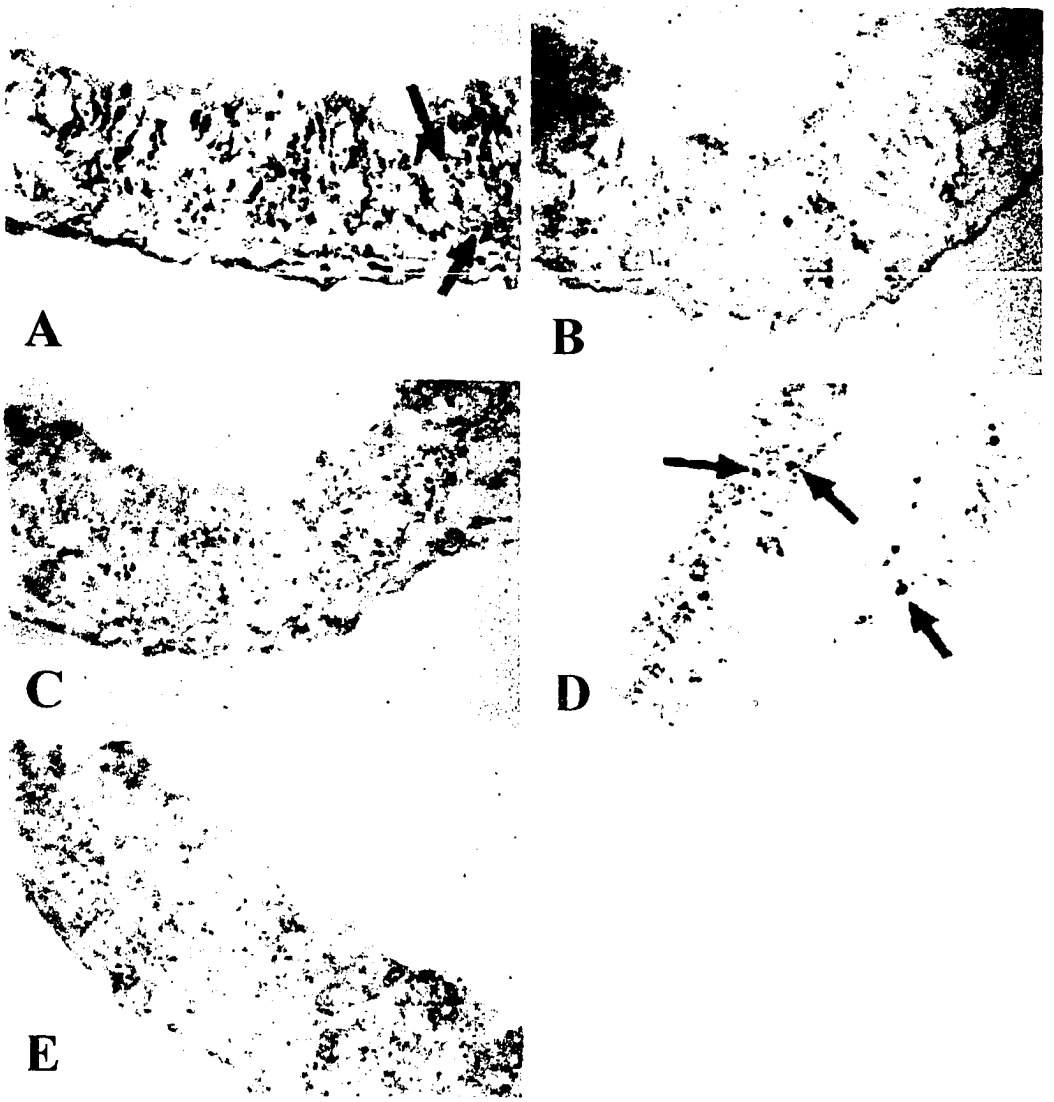


Figure 5

Figure 6. CP treatment causes no change in Cdk5 mRNA level.

A. Northern blot probed with ^{32}P-labeled Cdk5 RNA probe demonstrated an equal amount of Cdk5 mRNA in control (left, C) and CP (right, CP) embryos.

B. *In situ* hybridization with ^{35}S-labeled Cdk5 RNA antisense probe demonstrated that expression of Cdk5 mRNA is not confined to dying cells in control (a) and CP (b) embryos. 100x

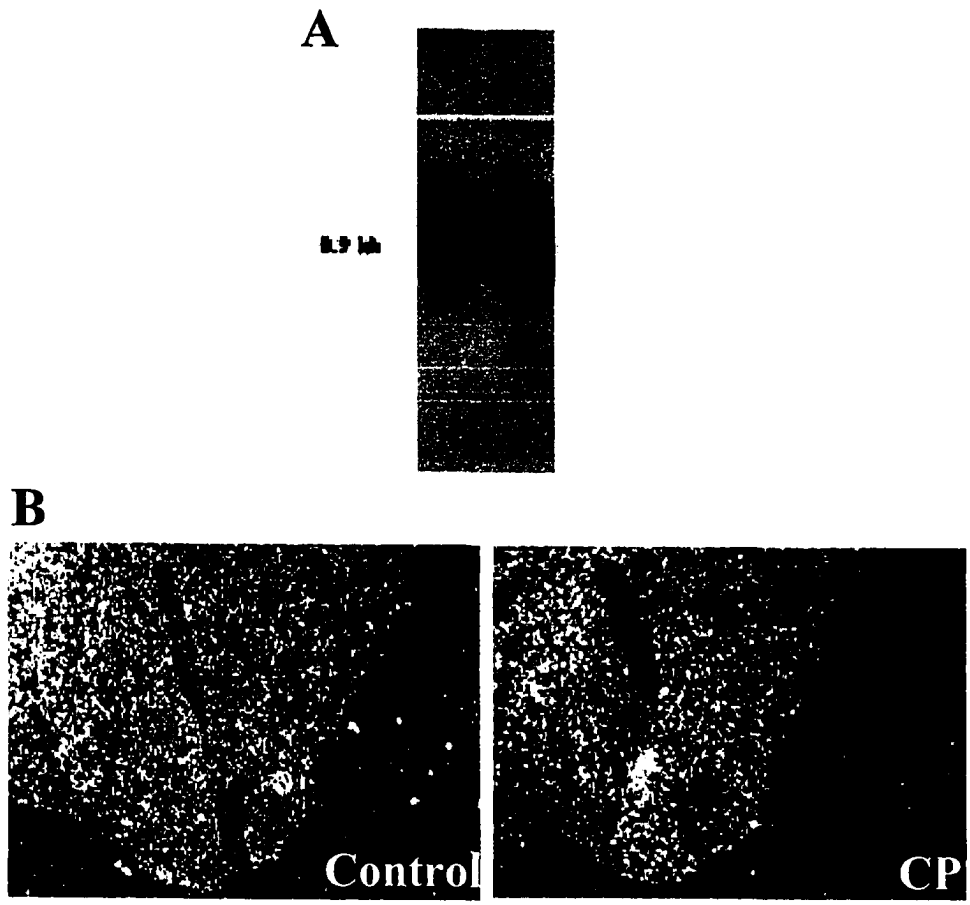


Figure 6

Figure 7. CP treatment causes no significant change in Cdk5 protein level detected by western blot. The up-regulation of Cdk5 in CP-treated embryos is not due to *de novo* Cdk5 protein synthesis.

A. Autoradiograph of total ^{35}S-methionine, cysteine incorporation in control and CP embryos for 4 hrs. Equal amounts of lysates from control and CP embryos were run on SDS-PAGE and blotted onto a nitrocellulose membranc, which was exposed to a film. The appearance of many bands indicated that many different proteins were labeled during the incorporation time in both control and CP embryos.

B. Immunoblot of equal amounts of lysates from control and CP embryos exposed to Cdk5 antibody. There was no change in the level of Cdk5 protein in CP embryos.

C. Autoradiograph of Cdk5 immunoprecipitated labeled proteins. Equal amounts of ^{35}S-labeled lysates from control and CP embryos were incubated with Cdk5 antibody for 1 hr, and the immunoprecipitates were run on SDS-PAGE and autoradiographed. The gel showed no radioactive signal, indicating no Cdk5 protein was synthesized during the 4 hr labeling time.

D. Western blot of the same Cdk5 immunoprecipitates as in C using Cdk5 antibody showed the presence of Cdk5 protein in the immunoprecipitates. The upper band (single arrow) was IgG. Cdk5 (double arrows) level was unchanged. In the above experiments, 12.5% acrylamide gel was used.

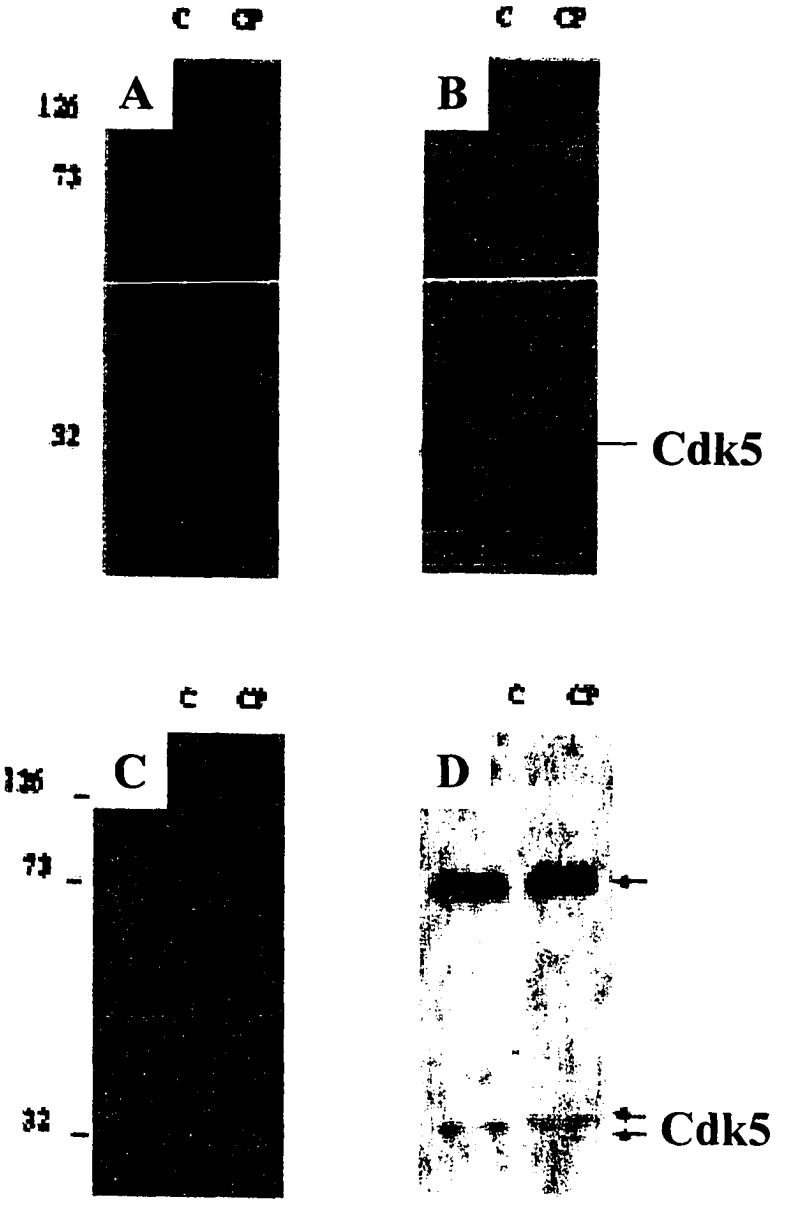


Figure 7

Figure 8. CP treatment causes a dramatic increase in Cdk5 kinase activity.

A. Histone H1 kinase activity in the Cdk5 immunoprecipitates from control and CP embryos. Cdk5 kinase activity of CP embryos rose substantially from undetectable level in control ones. 12.5% acrylamide gel was used.

B. Levels of Cdk5 kinase activity in control and CP embryos. There was a three-fold increase in Cdk5 kinase activity in CP embryos. The error bars represent the standard deviation from three trials of histone H1 kinase assay quantified by densitometry.

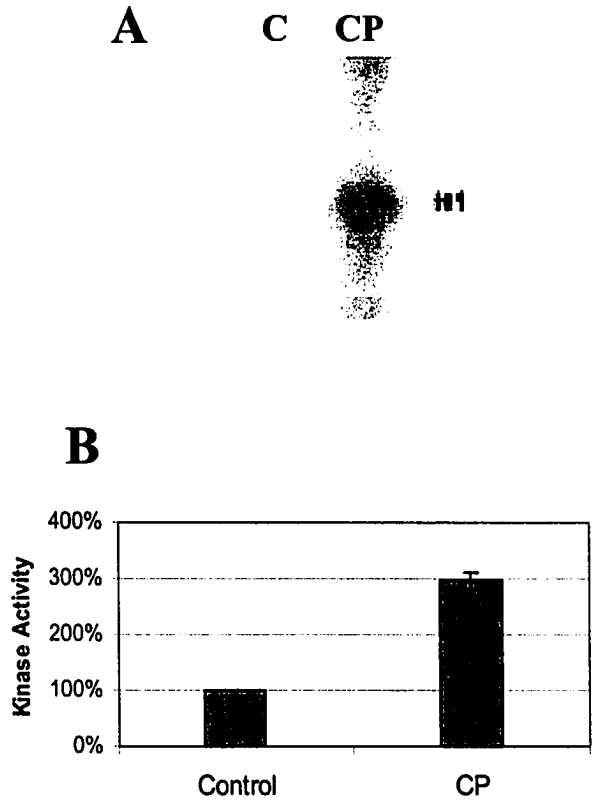


Figure 8

Figure 9. Cell death is induced in C8 (p53+/+) and A9 (p53-/-) cells by different stimuli.

Equal number of cells were plated in growth media 24 hrs before treatment and switched to media supplemented with 1% FBS for treatment. Cells were incubated with different death inducers, including ethanol (EtOH, 2.5%), cycloheximide (CHX, 25 μg/ml), and camptothecin (CPT, 20 μM), for 18 hrs. Levels of cell death were determined by trypan blue exclusion. A9 cells lacking p53 were more resistant to cell killing. The levels of cell death result from at least five independent trials of experiment, and error bars represent the standard deviation.

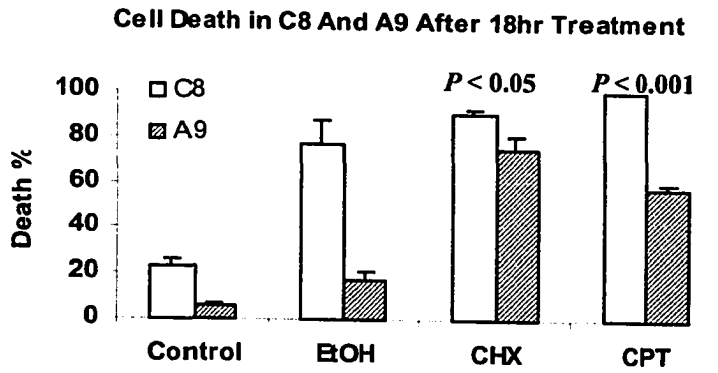


Figure 9

Figure 10. DNA ladder on agarose gel demonstrates that cell death induced in C8 and A9 is apoptotic.

C8 and A9 cells were incubated in 1% FBS media in the absence or presence of different insults, including EtOH, CHX and CPT, for 18 hrs. Low molecular weight DNA samples were isolated form C8 and A9 cells and run on 2% agarose gel. The DNA ladder resulted from internucleosomal DNA fragmentation is a hallmark of apoptosis. Lane 1-4, C8; lane 5-8, A9; lane 1 and 5, control; lane 2 and 6, CHX, 25 μg/ml; lane 3 and 7, EtOH, 2.5%; lane 4 and 8, CPT, 20 μM.

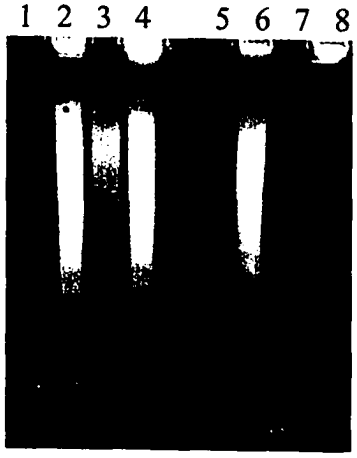


Figure 10

Figure 11. CPT-induced cell death in C8 and A9 is apoptotic shown by fragmented DNA and chromatin.

A-D, 18 hrs after CPT treatment, C8 and A9 cells were fixed with 3% paraformaldehyde, and DNA fragmentation was determined by TUNEL assay as described in Materials and Methods. TUNEL showed fragmented DNA in C8 (C) and A9 (D) cells treated with CPT for 18 hrs, compared with untreated C8 (A) and A9 (B) cells with normal morphological DNA. 400X.

E & F, CPT-treated C8 and A9 cells were fixed with 3% paraformaldehyde and stained with Hoechst fluorescent dye, which specifically binds to the nucleus. Hoechst staining exhibited condensed and fragmented nuclei in dying C8 (E) and A9 (F) cells. 400X.

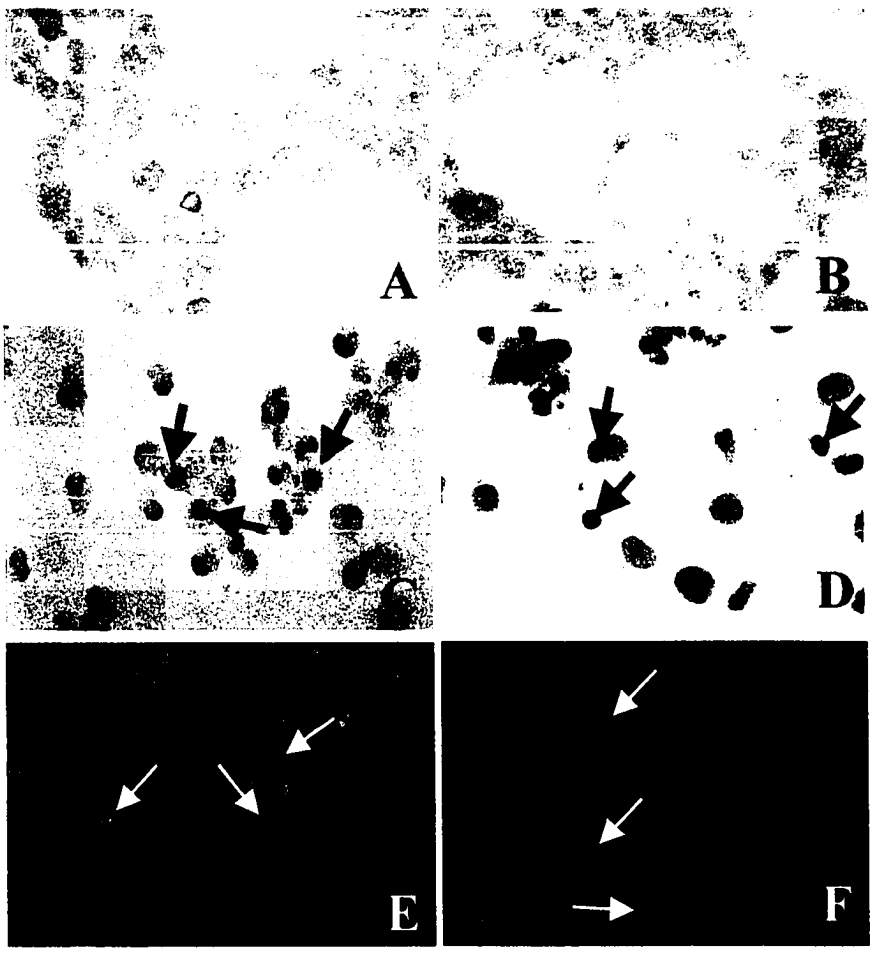


Figure 11

Figure 12. Activation of caspase-3 further demonstrates cell death induced in C8 and A9 is apoptotic.

C8 and A9 cells were incubated with different death inducers, including ethanol (EtOH, 2.5%), cycloheximide (CHX, 25 μg/ml), and camptothecin (CPT, 20 μM), for 18 hrs. Protein samples were isolated from C8 and A9 cells with RIPA buffer.

A. Western blot of equal amounts of lysates from C8 and A9 using active caspase-3 antibody demonstrated the induction of cleaved caspase-3, indicating activation of caspase-3 in cell death induced in C8 and A9. 15% acrylamide gel was used.
B. Western blot of equal amounts of lysates from C8 and A9 using PARP antibody exhibited the cleavage of PARP to form a 85 kDa fragment, indicating activation of caspase-3 in cell death induced in C8 and A9. 8% acrylamide gel was used.

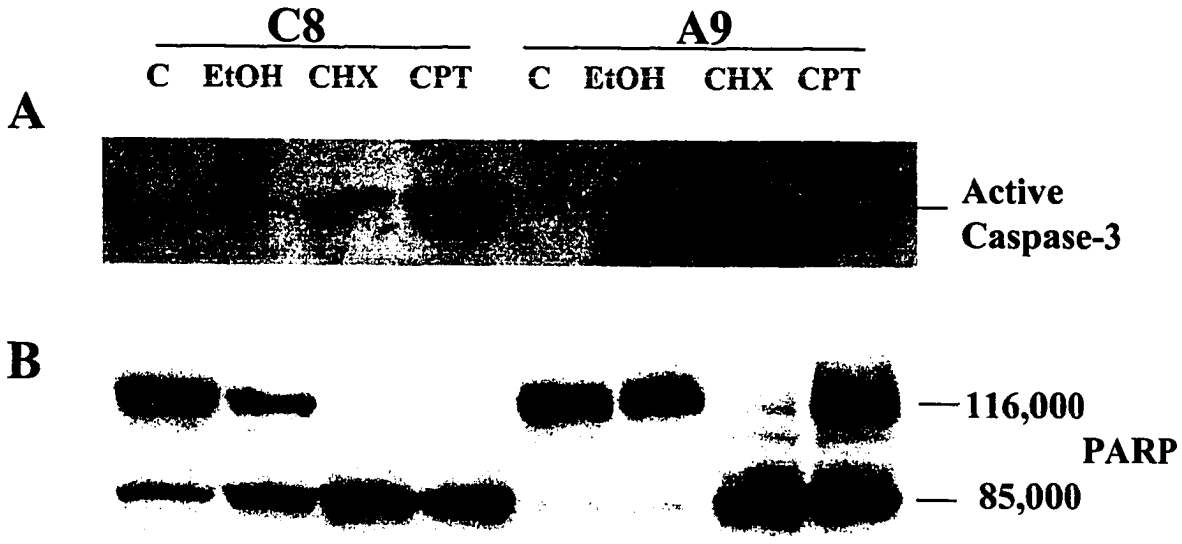


Figure 12

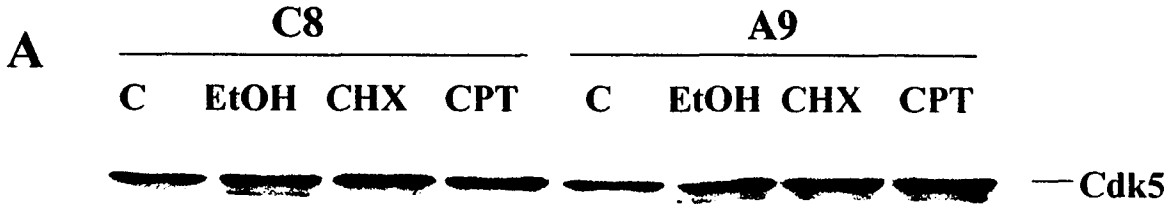
Figure 13. There is no change in levels of Cdk5 protein expression in cell death in both *in vivo* and *in vitro* systems.

A. There was no significant change in Cdk5 protein level in C8 and A9 cells treated with different stimuli. C8 and A9 cells were treated with different insults, including EtOH (2.5%), CHX (25μg/ml) and CPT (20μM) for 18 hr. Cells were collected and lysed with RIPA buffer. Equal amounts of lysates were loaded on 12.5% SDS-PAGE followed by evaluation of Cdk5 protein levels by western blot using Cdk5 antibody.

B. There was no change in the level and pattern of cell death and Cdk5 protein expression in wild type and p53-/- mouse embryos. Both wild type and p53-/- mouse embryos (ED 13.5) were fixed and sectioned as described in Materials and Methods. a and b, TUNEL assay on sections of wild type (a) and p53-/- (b) embryos showed cells with fragmented DNA in the brain, indicating that these cells die by apoptosis in this region. 400X.

c and d, immunohistochemistry on near serial sections of that in (a & b) using active caspase-3 antibody demonstrated cells with active caspase-3, further indicating there was apoptotic cell death in this region of wild type (c) and p53-/- (d) embryos.

e and f, immunohistochemistry on near serial sections of that in (a-d) using Cdk5 antibody exhibited cells expressing Cdk5 protein in the same brain region of wild type (e) and p53-/- (f) embryos. 400X.



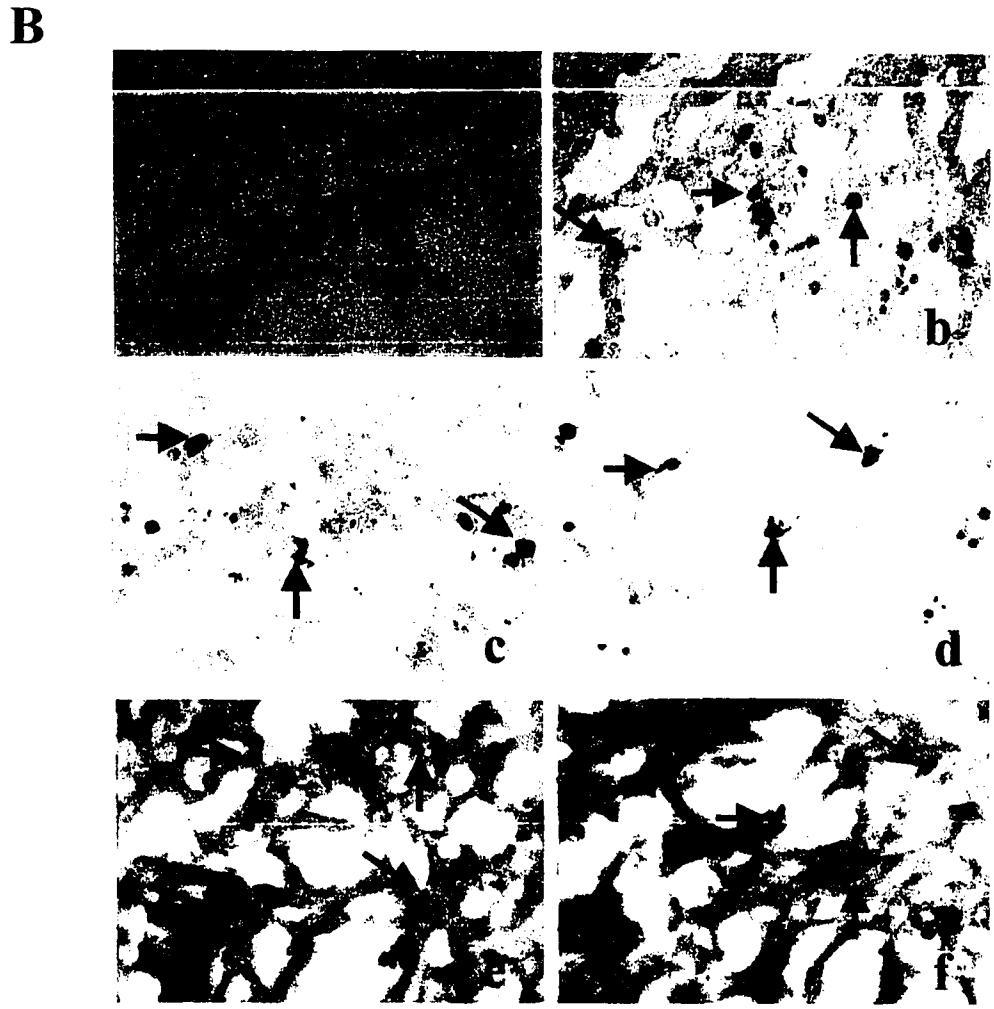


Figure 13

Figure 14. Activation of Cdk5 correlates with cell death induced in C8 and A9.

A. Histone H1 kinase activity of Cdk5 immunoprecipitates was induced in cell death induced in C8 and A9 cells. Following 18 hr treatment with EtOH (2.5%), CHX (25μg/ml) or CPT (20μM), C8 and A9 cells were collected and lysed with RIPA buffer. Kinase activity of Cdk5 immunoprecipitates was determined using histone H1 as *in vitro* substrate in the presence of $\gamma[^{32}P]$-ATP (for detail, see Materials and Methods). The incorporated $\gamma[^{32}P]$ in phosphorylated histone H1 was shown by exposing the gel onto a film. Compared with the control, cells induced to die by different stimuli showed differential levels of kinase activity corresponding to that of cell death. 12.5% acrylamide gel was used.

B. Levels of Cdk5 histone H1 kinase activity of (A) quantified by densitometry.

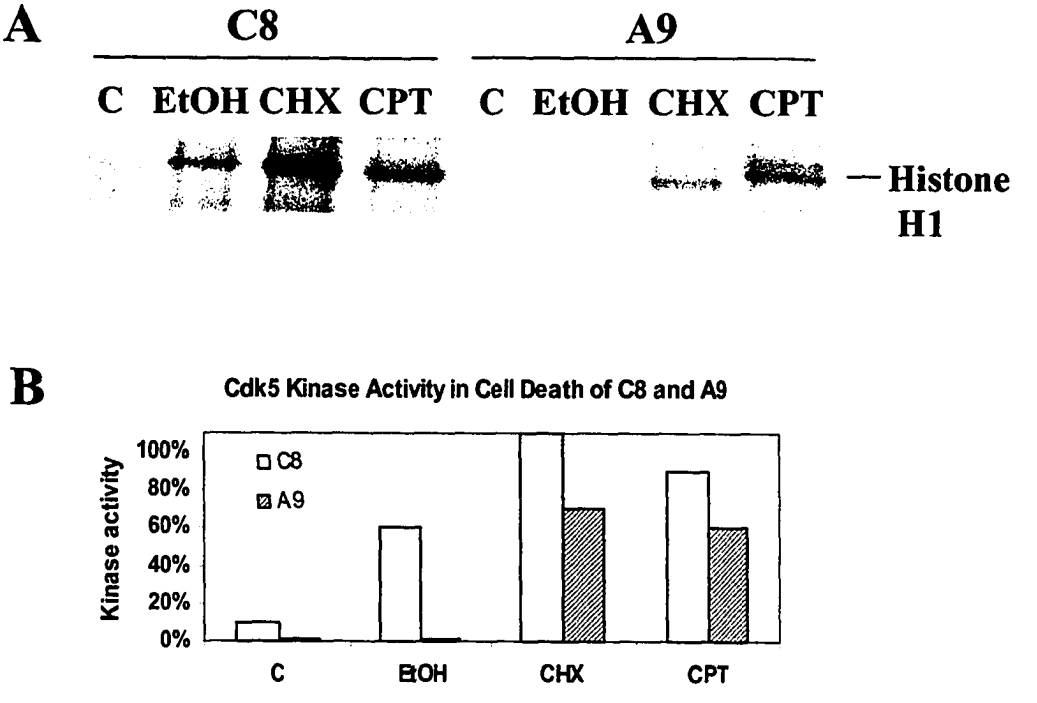


Figure 14

Figure 15. p25 is induced in cell death induced in C8 and A9.

A. Western blot of equal amounts of lysates from C8 and A9 cells treated with different stimuli (EtOH, CHX, and CPT) for 18 hrs, using p35 antibody, showed p25 is induced during cell death induced in both C8 and A9.

B. Levels of p25 induction of (A) quantified by densitometry.

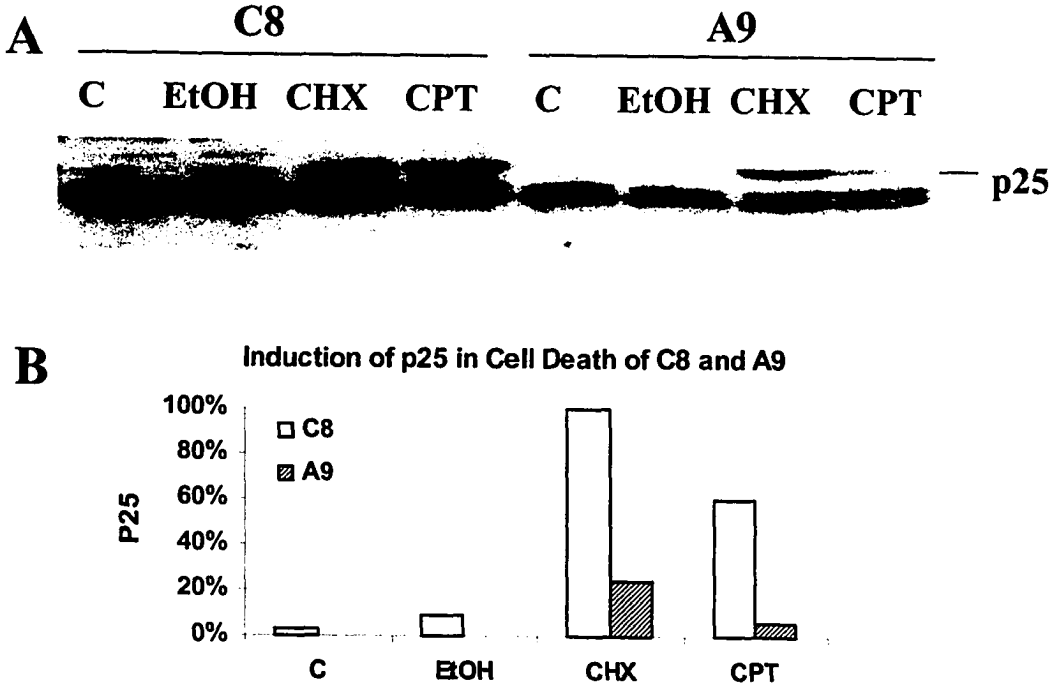


Figure 15

Figure 16. High transfection efficiency is achieved when sense or antisense p35 cDNA is introduced to C8 cells by retroviral vector pJim-IRES2-EGFP.

As described in Materials and Methods, C8 cells were incubated with viral supernatant overnight followed by culturing with normal growth media for 36 hrs.

A & C, C8 cells in phase contrast field 48 hrs after sense p35 (A) or antisense p35 (C) transfection. 250x

B & D, C8 cells transfected with sense p35 (B) or antisense p35 (D) fluorescence green 48 hrs after transfection. 250x

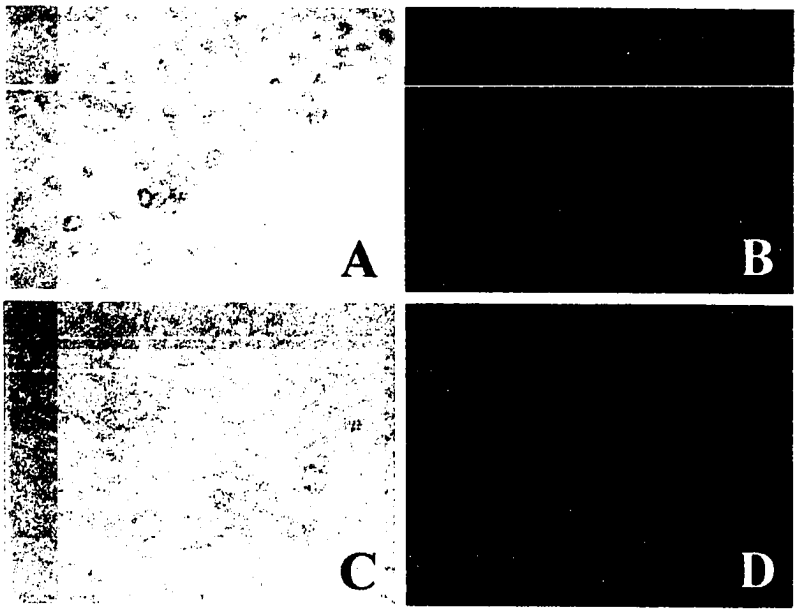


Figure 16

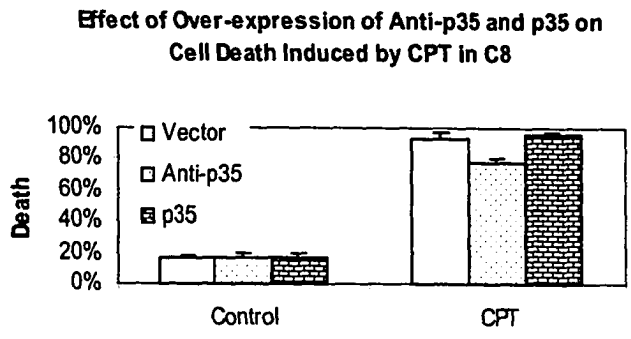
Figure 17. In C8 and A9 cells, blockage of p35 expression decreases CPT-induced cell death, coinciding with a reduction of p25 induction; over-expression of p35 increases CPT-induced cell death, corresponding to an increase of p25 induction.

C8 and A9 cells were transfected with either antisense or sense p35 retroviral constructs. 48 hrs after transfection, C8 and A9 cells with high transfection efficiency as detected in Figure 16 were treated with CPT for 18 hrs. Cells were collected for trypan blue cell death assay and lysed with RIPA buffer for western blot.

A & B, levels of CPT-induced cell death in C8 (A) or A9 (B) cells transfected with vector only, antisense or sense p35 by trypan blue exclusion.

C & D, western blot of equal amounts of lysates from C8 (C) or A9 (D) cells using p35 antibody demonstrated the induction of p25 during CPT-induced cell death in cells transfected with different constructs (vector, antisense p35, sense p35). 15% acrylamide gel was used.

A



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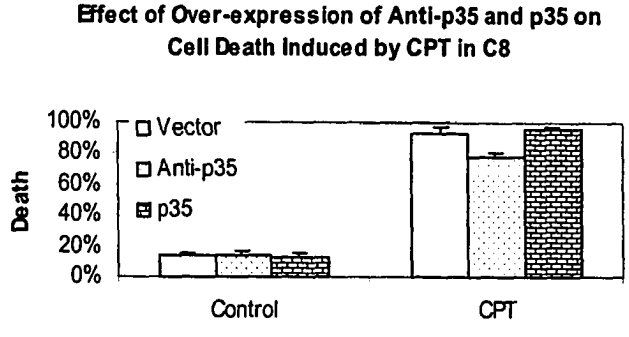

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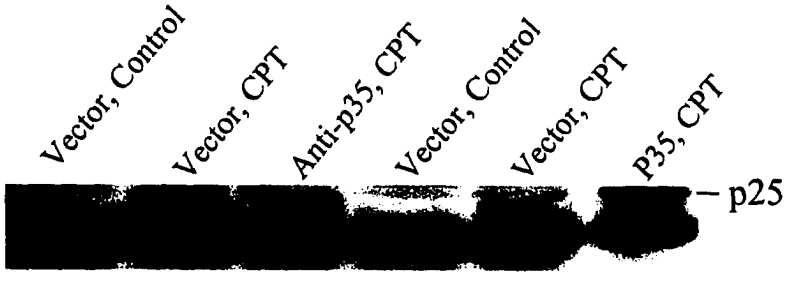

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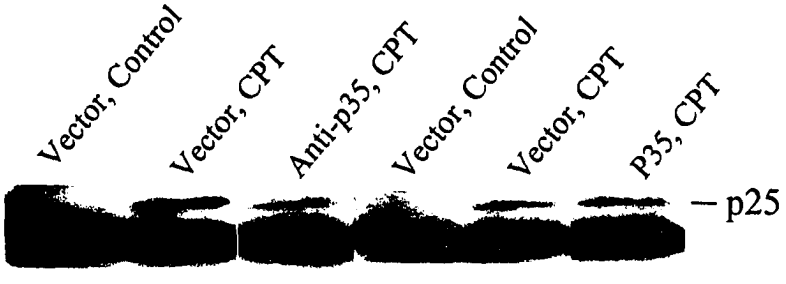


Figure 17

Figure 18. Calpain is activated in CP embryos.

Activation of calpain is determined by the production of specific spectrin breakdown products (SBDPs) at 150 kDa and 145 kDa resulted from cleavage of non-erythroid α-spectrin by active calpain. Western blot of equal amounts of lysates from control and CP embryos using spectrin antibody. The dual bands at 145 kDa and 150 kDa indicated activation of calpain in CP embryos, compared to no detectable level of calpain activity in control embryos. 8% acrylamide gel was used.

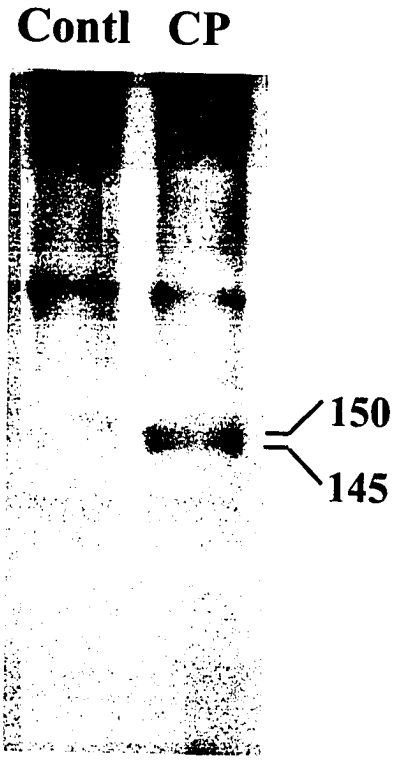


Figure 18

Figure 19. Calpain is activated during cell death induction in both C8 and A9 cells.

C8 and A9 cells were treated with different insults, including EtOH (2.5%), CHX (25μg/ml) and CPT (20μM) for 18 hr. Either of the two calpain inhibitors, calpastatin (10 μM) or PD150606 (10 μM), was coadministered with CPT to C8 and A9 cells for 18 hrs. Cells were lysed with RIPA buffer. Production of specific spectrin breakdown products (SBDPs) at 150 kDa and 145 kDa resulted from cleavage of non-erythroid α-spectrin by calpain was used to examine calpain activity.

A. Western blot of equal amounts of lysates from C8 and A9 of different treatment (EtOH, CHX or CPT) using spectrin antibody showed the appearance of dual bands at 145 kDa and 150 kDa when cell death was induced in both C8 and A9 cells, indicating calpain is activated. 8% acrylamide gel was used.

B & C, western blot of equal amounts of lysates from C8 and A9 cells treated with CPT in the presence or absence of CS (B) or PD (C) using p35 antibody demonstrated a disappearance of p25 induction in CPT killing. CPT-induced cell death was reduced upon calpain inhibition by CS (B) or PD (C) in C8 and A9 measured by trypan blue.

D. Western blot of equal amounts of lysates from C8 and A9 cells treated with CPT in the presence or absence of CS or PD using spectrin antibody demonstrated a disappearance of 145- and 150-kDa dual bands in CPT killing, further confirming that CS or PD can inhibit calpain activation.

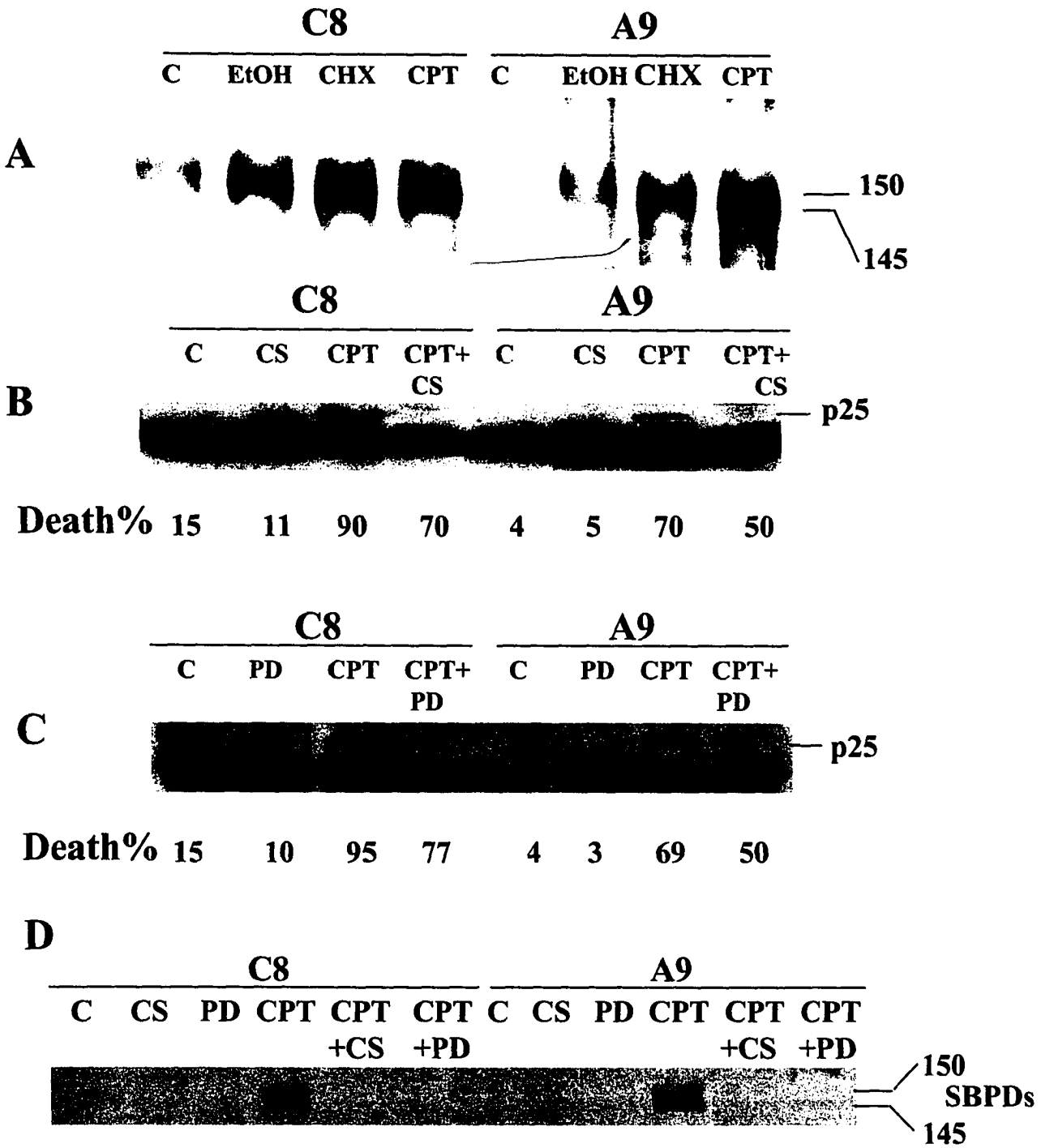


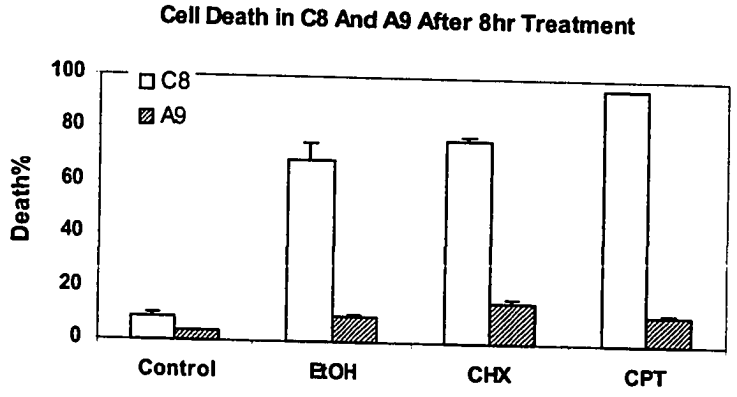
Figure 19

Figure 20. There is a delayed induction of cell death in A9 cells compared to C8.

A. There were substantial levels of cell death in C8 cells treated with different stimuli (EtOH, CHX or CPT) for 8 hrs, compared to insignificant levels of cell death in A9 cells, indicating a delayed onset of cell death due to lack of p53 in A9. C8 and A9 cells were treated with different insults, including EtOH (2.5%), CHX (25μg/ml) and CPT (20μM) for 8 hrs. Levels of cell death were determined by trypan blue exclusion. The error bars represent standard deviations obtained from at least 3 trials of experiments.

B. Kinetic study of cell death induced by CPT in C8 and A9 showed that there was a delayed cell death induction in A9 cells treated with CPT. C8 and A9 cells were incubated with CPT (20μM), and cell death level was determined at different times (0, 3, 6, 8, 12, 15, and 18 hr) by trypan blue exclusion. The error bars represent standard deviations obtained from at least 3 trials of experiments.

A



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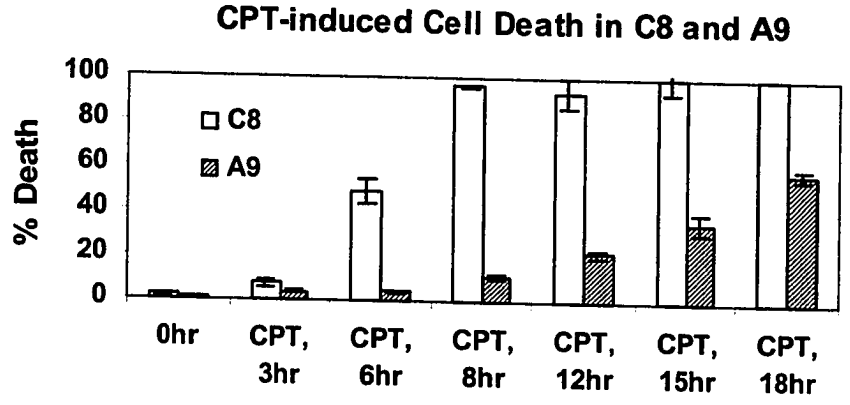

Figure 20

Figure 21. There is a delayed activation of caspase-3, Cdk5 and calpain in CPT-induced cell death in A9 cells.

C8 and A9 cells were treated with CPT (20μM), and at different time points (0 hr, 3 hr, 6 hr, and 12 hr), cells were collected and lysed in RIPA buffer. Equal amounts of cell lysates were run on 8% SDS-PAGE.

A. Western blot of equal amounts of lysates from C8 and A9 at different time points using Cdk5 antibody exhibited an unchanged level of Cdk5 protein.

B. Western blot of equal amounts of lysates from C8 and A9 at different time points using active caspase-3 antibody demonstrateed a delayed activation of caspase-3 in A9 cells treated with CPT.

C. Western blot of equal amounts of lysates from C8 and A9 at different time points using p35 antibody exhibited a delayed induction of p25, indicative of activation of Cdk5, in A9 cells treated with CPT.

D. Western blot of equal amounts of lysates from C8 and A9 at different time points using spectrin antibody demonstrated a delayed activation of calpain in A9 cells treated with CPT.

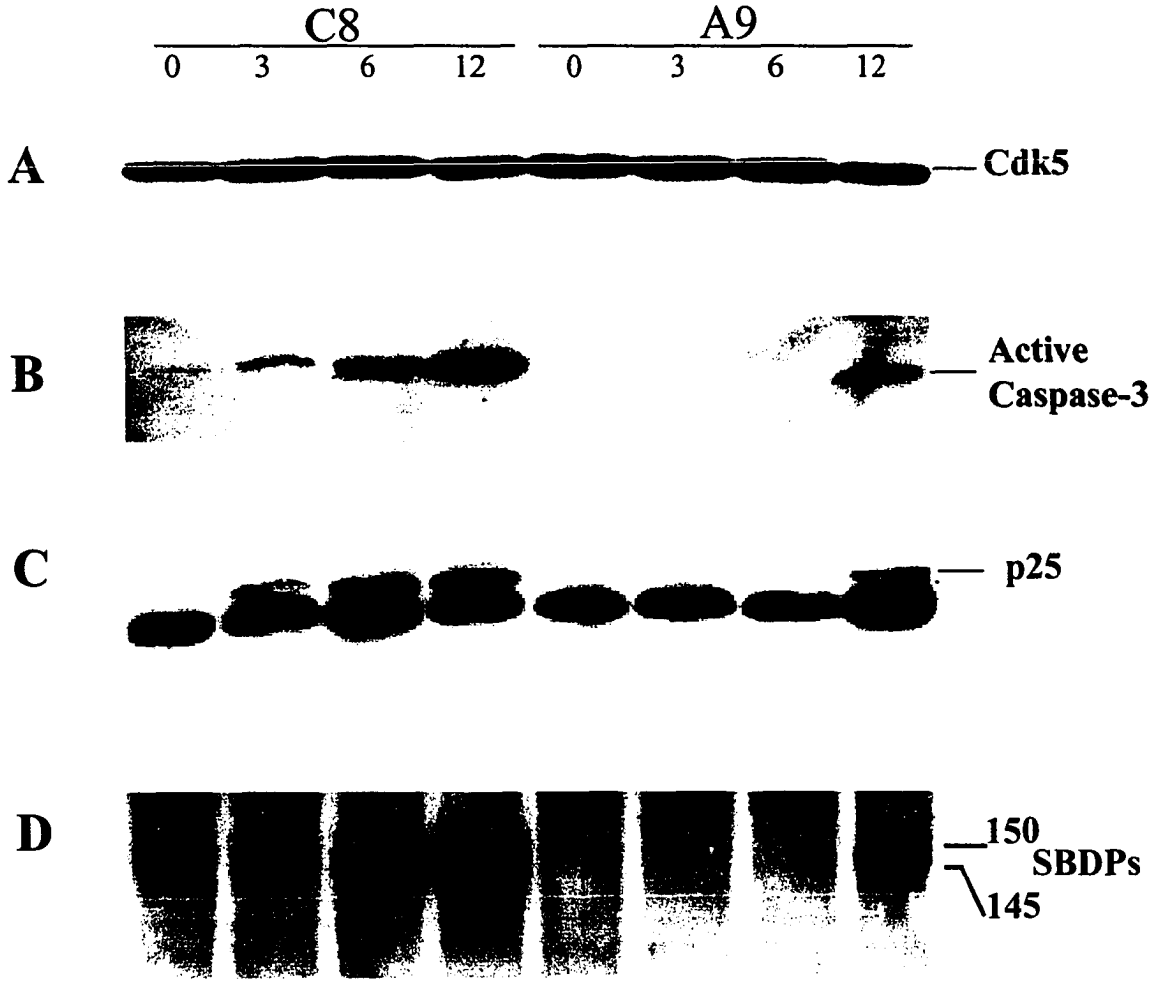

Figure 21

Figure 22. Kinetic study of cell death induced by UV in C8 and A9 shows that there is a delayed cell death in A9 treated with UV.

C8 and A9 cells were irradiated with 254 nm UV (50 J/m2) followed by incubating in 1% FBS media. At different time points (0, 8, 12 and 18 hr), levels of cell death in both cells were determined by trypan blue exclusion. The error bars represent the standard deviations from at least three individual experiments.

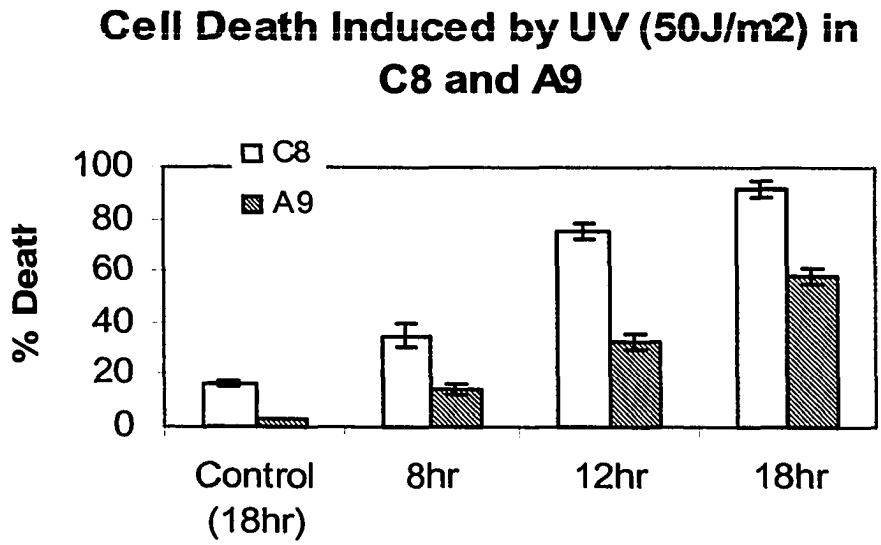

Figure 22

Figure 23. There is a delayed activation of caspase-3 and Cdk5 in UV-treated A9 cells compared to C8.

C8 and A9 cells were irradiated with 254 nm UV (50 J/m2) followed by incubation in 1% FBS media. At different times (0, 8, 12 and 18 hr), both cells were collected and lysed with RIPA buffer for western blot.

A. Western blot of equal amounts of lysates from C8 and A9 cell samples at different times using Cdk5 antibody demonstrated that there was no change in Cdk5 protein level during cell death induced by UV. 12.5% acrylamide gel was used.

B. Western blot of equal amounts of lysates from C8 and A9 cell samples at different times using active caspase-3 antibody demonstrated that there was a delayed activation of caspase-3 in A9 cells treated with UV. 15% acrylamide gel was used.

C. Western blot of equal amounts of lysates from C8 and A9 cell samples at different time points using p35 antibody demonstrated that there was a delayed induction of p25, indicative of a delayed activation of Cdk5, in A9 cells treated with UV. 15% acrylamide gel was used.

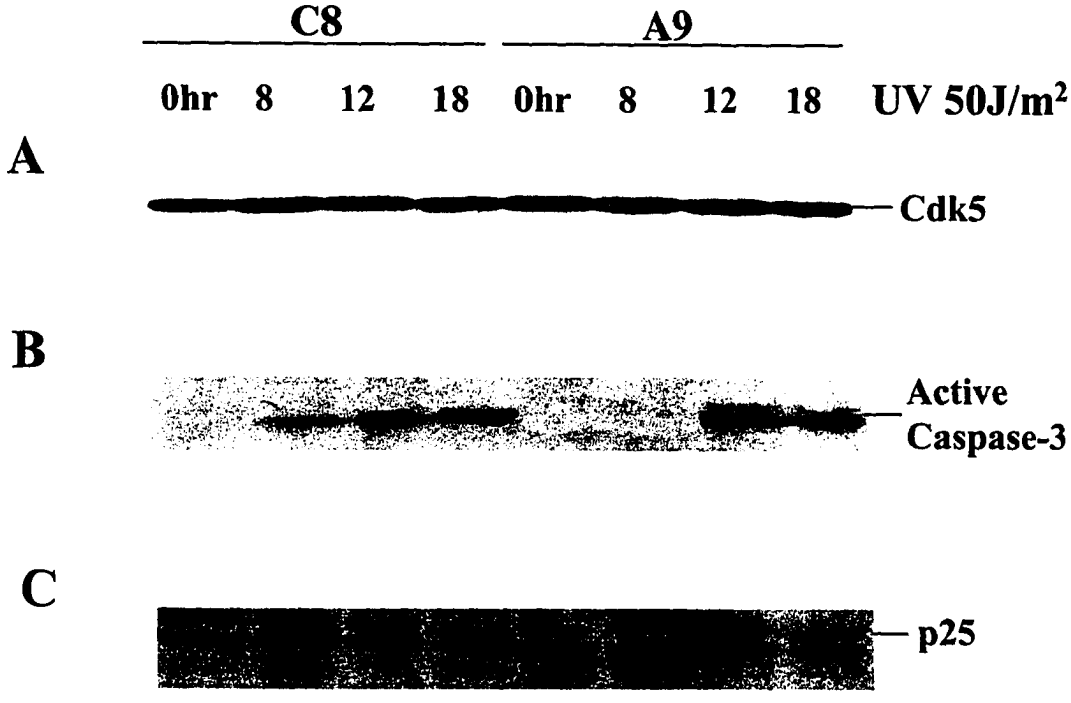

Figure 23

Figure 24. Cell death and Cdk5 expression in the periphery of liver of Apaf-1 knockout mouse embryos (ED 13.5).

Apaf-1 knockout mouse embryos (ED 13.5) were fixed and sectioned as described in Materials and Methods.

A. TUNEL assay demonstrated cells with fragmented DNA in the periphery of liver in Apaf-1 knockout embryo, indicating that these cells are undergoing cell death. 400X.

B. Immunohistochemistry of an adjacent section of (A) using Cdk5 antibody showed cells expressing Cdk5 protein in the same region, indicating that Apaf-1 is not required for Cdk5 expression. 400X.

Figure 24

Figure 25. Cdk5 is activated during cell death in both wild type and Apaf-1-/- MEFs.

Wild type and Apaf-1-/- MEFs were treated with CHX (25 μg/ml) or CPT (20 μM) for 45 hrs. Levels of cell death were measured by trypan blue and cells were lysed with RIPA buffer for western blot.

A. Cell death was induced in both wild type and Apaf-1-/- MEFs by CHX or CPT, measured by trypan blue.

B. Western blot of equal amounts of lysates from wild type and Apaf-1-/- MEFs with different treatments (untreated control, 25 μg/ml CHX, 20 μM CPT) using Cdk5 antibody, showed an unchanged level of Cdk5 protein. 12.5% acrylamide gel was used.

C. Western blot of equal amounts of lysates from wild type and Apaf-1-/- MEFs with different treatments (untreated control, 25 μg/ml CHX, 20 μM CPT) using p35 antibody, showed an induction of p25 in both cells when cell death was induced, indicative of the activation of Cdk5. 15% acrylamide gel was used.

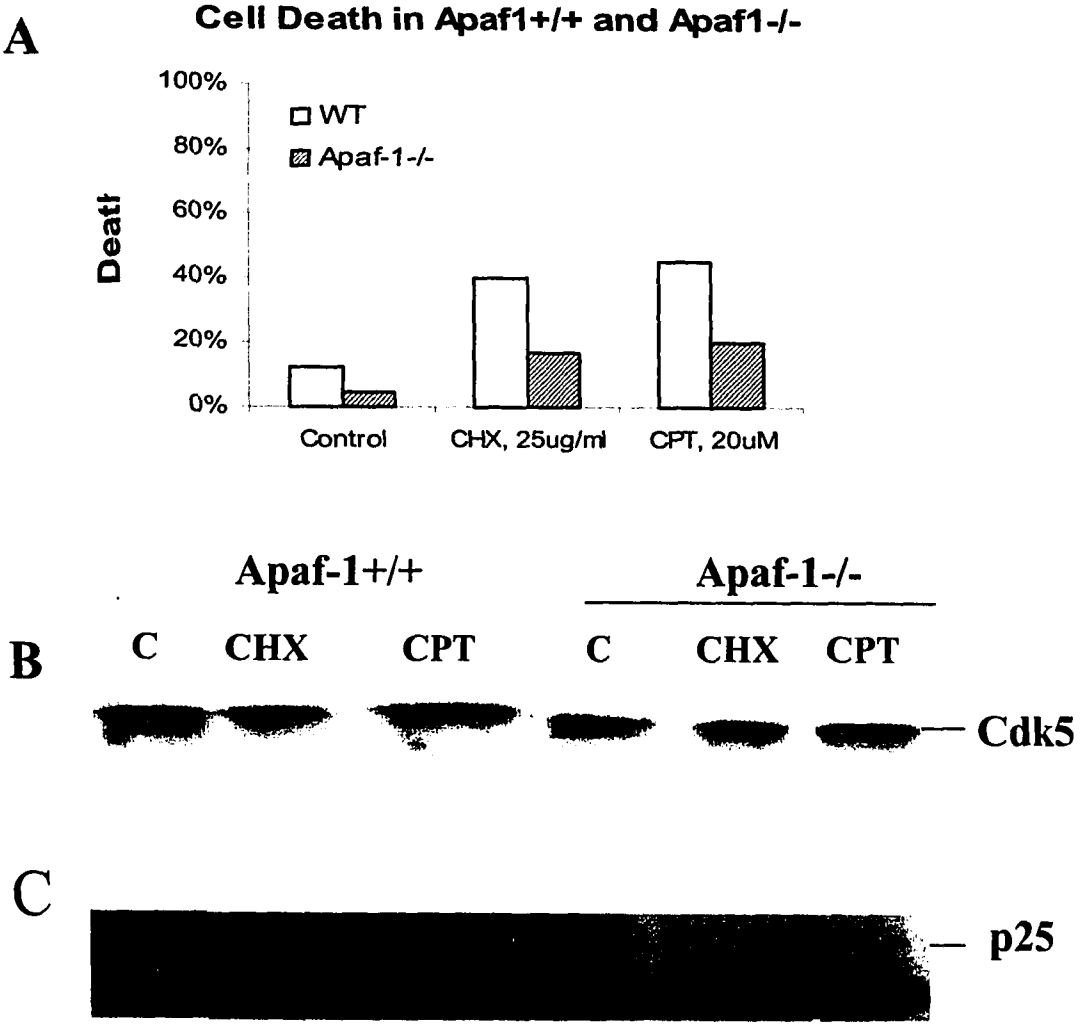

Figure 25

Figure 26. Cdk5 is activated during cell death in both wild type and caspase-9-/- MEFs.

Wild type and caspase-9-/- MEFs were treated with CHX (25 μg/ml) or CPT (20 μM) for 24 hrs. Levels of cell death were measured by trypan blue and cells were lysed with RIPA buffer for western blot.

A. Cell death is induced in both wild type and caspase-9-/- MEFs by CHX or CPT, measured by trypan blue. Caspase-9-/- cells are more resistant to cell death induced by CHX or CPT, indicated by the much lower levels of cell death induced ($p<0.05$). The error bars represent standard deviations obtained from 3 independent experiments.

B. Western blot of equal amounts of lysates from wild type and caspase-9-/- MEFs with different treatments (untreated control, 25 μg/ml CHX, 20 μM CPT) using Cdk5 antibody, shows an unchanged level of Cdk5 protein. 12.5% acrylamide gel was used.

C. Western blot of equal amounts of lysates from wild type and caspase-9-/- MEFs with different treatments (untreated control, 25 μg/ml CHX, 25 μM CPT) using p35 antibody, showed an induction of p25 in both cells when cell death was induced, indicative of the activation of Cdk5. 15% acrylamide gel was used.

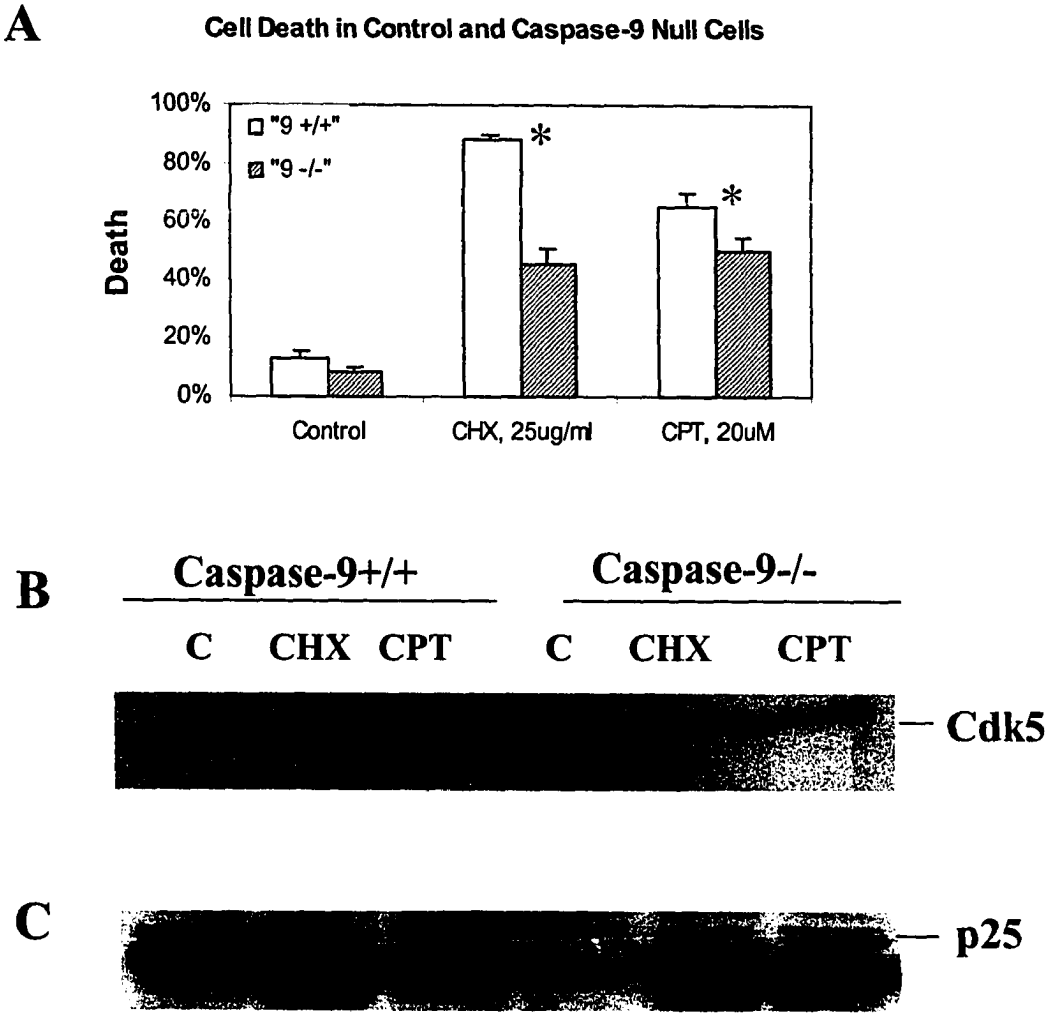

Figure 26

Figure 27. Cell death and Cdk5 expression in brain of caspase-3 knockout mouse embryos (ED 13.5).

Caspase-3 knockout mouse embryos (ED 13.5) were fixed and sectioned as described in Materials and Methods.

A. TUNEL assay demonstrated cells with fragmented DNA in brain region in caspase-3 knockout embryo, indicating these cells undergo caspase-3-independent cell death. 400X.

B. Immunohistochemistry of an adjacent section of (A) using Cdk5 antibody showed cells expressing Cdk5 protein in the same brain region, indicating that caspase-3 is not required for Cdk5 expression during cell death. 400X.

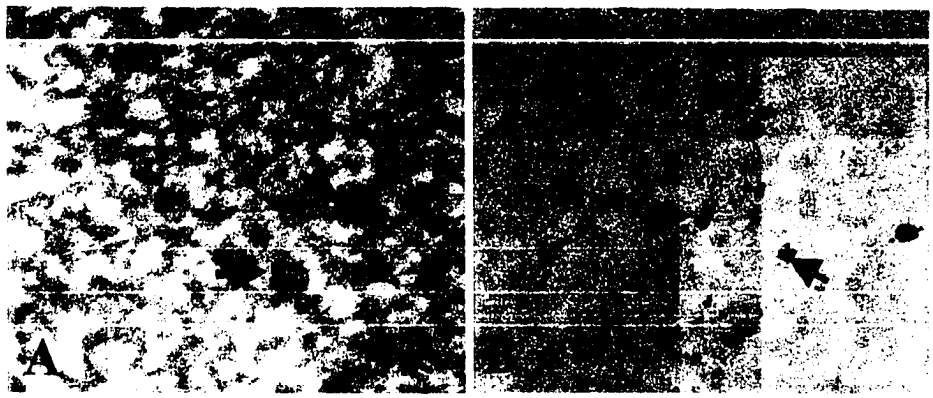

Figure 27

Figure 28. Cell death is induced in wild type and caspase-3 -/- primary mouse embryonic fibroblasts (MEFs) by CHX or CPT.

Wild type and caspase-3-/- MEFs were treated with CHX (25 μg/ml) or CPT (20 μM) for 24 hrs, and levels of cell death were measured by trypan blue exclusion. Caspase-3-/- cells are more resistant to cell death induction. The error bars represent standard deviations obtained from 3 independent experiments.

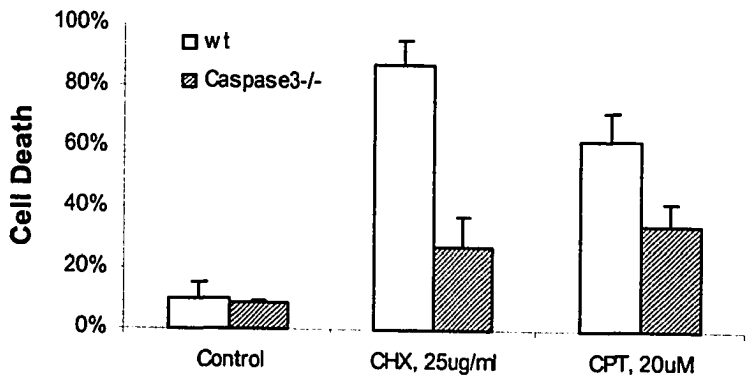

Figure 28

Figure 29. There is an induction of p25 and activation of Cdk5 during cell death induced in both wild type and caspase-3-/- MEFs.

Wild type and caspase-3-/- MEFs were treated with CHX (25 μg/ml) or CPT (20 μM) for 24 hrs, and cells were lysed with RIPA buffer.

A. Western blot of equal amounts of lysates from wild type and caspase-3-/- MEFs using Cdk5 antibody showed an unchanged level of Cdk5 protein during cell death. 12.5% acrylamide gel was used.

B. Western blot of equal amounts of lysates from wild type and caspase-3-/- MEFs using p35 antibody demonstrated an induction of p25 when cell death is induced in both MEFs. 15% acrylamide gel was used.

C. Histone H1 kinase activity of Cdk5 immunoprecipitates was induced in cell death induced in wild type and caspase-3-/- MEFs. Following 24 hr treatment with CHX (25μg/ml) or CPT (20μM), cells were collected and lysed with RIPA buffer. Kinase activity of Cdk5 immunoprecipitate was determined using histone H1 as *in vitro* substrate in the presence of $\gamma[^{32}P]$-ATP (for detail, see Materials and Methods). The incorporated $\gamma[^{32}P]$ in phosphorylated histone H1 was shown by exposing the gel to a film. Compared with the control, cells induced to die by different stimulus showed different levels of kinase activity corresponding to that of cell death. 12.5% acrylamide gel was used.

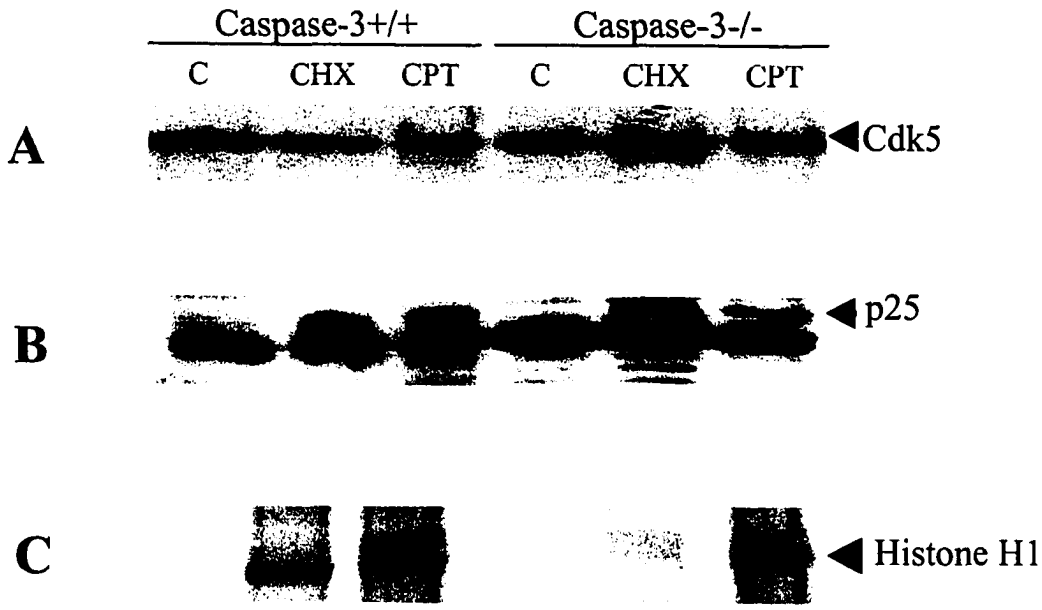

Figure 29

Figure 30. Inhibition of caspases activation by zVAD-fmk reduces cell death induced by CPT in C8.

C8 cells were treated with CPT with or without addition of 50 μM zVAD-fmk for 18 hrs. Levels of cell death were measured by trypan blue. The error bars represent standard deviations obtained from 3 independent experiments.

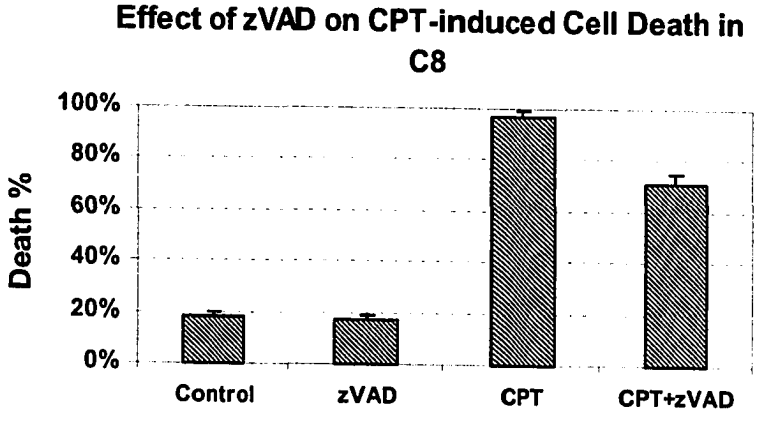

Figure 30

Figure 31. Inhibition of caspases activity does not block Cdk5 activation during cell death.

C8 cells were treated with 20 μM CPT with or without addition of 50 μM zVAD-fmk for 18 hrs. Cells were lysed with RIPA buffer.

A. Western blot of equal amounts of lysates from C8 with different treatments (untreated control, zVAD only, CPT only, CPT and zVAD) using active caspase-3 antibody, demonstrated that zVAD inhibits caspase-3 activation during CPT killing. 15% acrylamide gel was used.

B. Western blot of equal amounts of lysates from C8 with different treatments (untreated control, zVAD only, CPT only, CPT and zVAD) using Cdk5 antibody, showed an unchanged level of Cdk5 protein. 12.5% acrylamide gel was used.

C. Western blot of equal amounts of lysates from C8 with different treatments (untreated control, zVAD only, CPT only, CPT and zVAD) using p35 antibody, exhibited an induction of p25 when caspases are inhibited by zVAD in CPT killing, indicating Cdk5 activation is caspases-independent during cell death. 15% acrylamide gel was used.

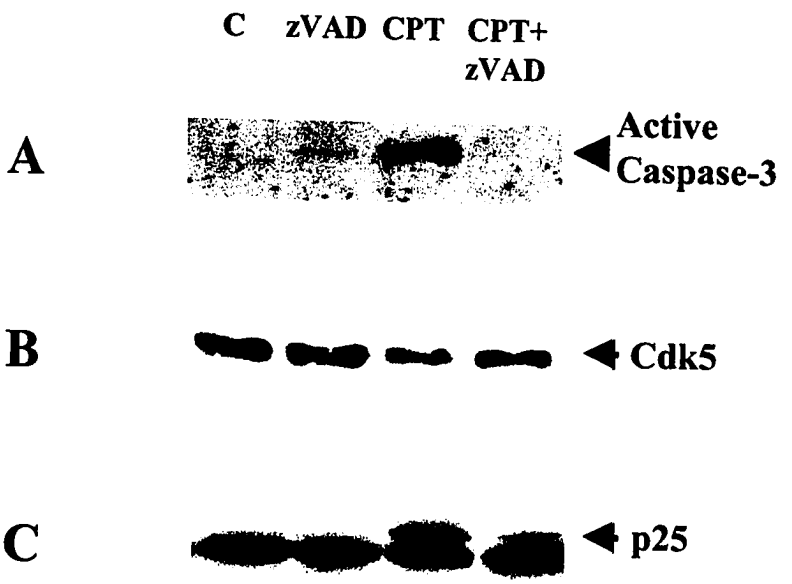

Figure 31

REFERENCES

Abdelwahid E, Pelliniemi LJ, Jokinen E. 2002. Cell death and differentiation in the development of the endocardial cushion of the embryonic heart. Microsc Res Tech., 58(5):395-403.

Ahlijanian MK, Barrezueta NX, Williams RD, Jakowski A, Powsz KP, McCarthy S, Coskran T, Carlo A, Seymour PA, Burkhardt JE, Nelson RB, & McNeish JD. 2000. Hyperphosphorylated tau and neurofilament and cytoskeletal disruptions in mice overexpressing human p25, an activator of Cdk5. Proc. Natl. Acad. Sci. USA, 97(6): 2910-2915.

Ahmad N, Cheng P, & Mukhtar H. 2000. Cell cycle dysregulation by green tea polyphenol epigallocatechin-3-gellate. Biochem. Biophys. Res. Commun., 275(2): 328-334.

Ahuja HS, James W and Zakeri Z. 1997. Rescue of the limb deformity in Hammertoe mutant mice by retinoic acid-induced cell death. Dev. Dynamics 208: 1-16

Ahuja HS, Zhu Y and Zakeri Z. 1997. Association of cyclin-dependent kinase 5 and its activator p35 with apoptotic cell death. Dev. Genet. 21: 258-267.

Alvarez A, Muñoz JP, & Maccioni RB. 2001. A Cdk5-p35 stable complex is involved in the β-amyloid-induced deregulation of Cdk5 activity in hippocampal neurons. Exp. Cell Res., 264: 266-274.

Alvarez A, Toro R, Cáceres A, & Maccioni RB. 1999. Inhibition of tau phosphorylating protein kinase cdk5 prevents beta-amyloid induced neuronal death. FEBS Lett., 459: 421-426.

Anderson AJ, Su JH, Cotman CW. 1996. DNA damage and apoptosis in Alzheimer's disease: colocalization with c-Jun immunoreactivity, relationship to brain area, and effect of postmortem delay. J. Neurosci., 16: 1710-1719.

Andrade F, Roy S, Nicholson D, Thornberry N, Rosen A, & Casciola-Rosen L. 1998. Granzyme B directly and efficiently cleaves several downstream caspase substrates implications for CTL-induced apoptosis. Immunity, 8: 451-460.

Anglade P, Vyas S, Javoy-Agid P, et al. 1997. Apoptosis and autophagy in nigral neurons of patients with Parkinton's disease. Histol. Histppathol., 12: 25-31.

Ankarcrona M, Dypbukt JM, Bonfoco E, Zhivotovsky B, Orrenius S, Lipton SA, & Nicotera P. 1995. Glutamate-induced neuronal death: a succession of necrosis or apoptosis depending on mitochondrial function. Neuron, 15: 961-973.

Arase H, Lanier LL. 2002. Virus-driven evolution of natural killer cell receptors. Microbes. Infect., 4(15): 1505-12.

Arredondo J, Nguyen VT, Chernyavsky AI, Bercovich D, Orr-Urtreger A, Vetter DE, Grando SA. 2003. Functional role of alpha7 nicotinic receptor in physiological control of cutaneous homeostasis. Life Sci., 72(18-19): 2063-7.

Artandi SE. 2002. Telomere shortening and cell fates in mouse models of neoplasia. Trends Mol. Med., 8(1): 44-7.

Atienza C Jr, Elliott MJ, Dong YB, Yang HL, Stilwell A, Liu TJ, & McMasters KM. 2000. Adenovirus-mediated E2F-1 gene transfer induces an apoptotic response in human gastric carcinoma cells that is enhanced by cyclin dependent kinase inhibitors. Int. J. Mol. Med., 6(1): 55-63.

Avallone B, Balsamo G, Trapani S, Marmo F. 2002. Apoptosis during chick inner ear development: some observations by TEM and TUNEL techniques. Eur J Histochem., 46(1):53-9.

Ballard KJ, Holt SJ. 1968. Cytological and cytochemical studies on cell death and digestion in the foetal rat foot: the role of macrophages and hydrolytic enzymes. J. Cell Sci., 3: 245-62.

Bao JJ, Le XF, Wang RY, Yuan J, Wang L, Atkinson EN, LaPushin R, Andreeff M, Fang B, Yu Y, Bast RC Jr. 2002. Reexpression of the tumor suppressor gene ARHI induces apoptosis in ovarian and breast cancer cells through a caspase-independent calpain-dependent pathway. Cancer Res., 62(24): 7264-72.

Barres BA, & Raff MC. 1999. Axonal control of oligodendrocyte development. J. Cell Biol., 147: 1123-1128.

Bartke T, Siegmund D, Peters N, Reichwein M, Henkler F, Scheurich P, & Wajant H. 2001. p53 upregulates cFLIP, inhibits transcription of NF-kappaB-regulated genes and induces caspase-8-independent cell death in DLD-1 cells. Oncogene, 20(5): 571-580.

Baker SJ, Markowitz S, Fearon ER, Willson JKV, & Vogelstein B. 1990. Suppression of human colorectal carcinoma cell growth by wild-type p53. Science, 249: 912-915.

Baldwin RL, Tran H, Karlan BY. 2003. Loss of c-myc repression coincides with ovarian cancer resistance to transforming growth factor beta growth arrest independent of transforming growth factor beta/smad signaling. Cancer Res., 63(6): 1413-1419.

Banin S, Moyal L, Khostravi R, Shieh SY, Taya Y, Anderson CW, Chessa L, Smorodinsky NI, Prives C, Shiloh Y, Ziv Y. 1998. Enhanced phosphorylation of p53 by ATM in response to DNA damage. Science, 281: 1674-7.

Barley RD, Enns L, Paterson MC, Mirzayans R. 1998. Aberrant p21WAF1-dependent arrest as the possible mechanism of abnormal resistance to ultraviolet light cytotoxicity in

Li-Fraumeni syndrome fibroblast strains heterozygous for TP53 mutation. Oncogene, 17(5): 533-43.

Beaudette K, Lew J, & Wang JH. 1993. Substrate specificity characterization of a Cdc2-like protein kinase purified from bovine brain. J. Biol. Chem., 268: 20825-20830.

Bednarski E, Vanderklish P, Gall C, Saido T, Bahr B, and Lynch G. 1995. Translational suppression of calpain I reduces NMDA-induced spectrin proteolysis and pathophysiology in cultured hippocampal slices. Brain Res., 694: 147-157.

Behl C, Davis JB, Lesley R, & Schubert D. 1994. Hydrogen peroxide mediates amyloid β protein toxicity. Cell, 77: 817-827.

Beilharz EJ, Williams CE, Dragunow M, Sirimanne ES, and Gluckman PD. 1995. Mechanisms of delayed cell death following hypoxia-ischemic injury in the immature rat: evidence for apoptosis during selective neuronal loss. Brain Res. Mol. Brain Res., 29: 1-14.

Beresford, P.J. *et al.* 2001. Granzyme A activates an endoplasmic reticulum-associated caspaseindependent nuclease to induce single-stranded DNAnicks. J. Biol. Chem., 276: 43285–93.

Berg CP, et al. 2001. Human mature red blood cells express caspase-3 and caspase-8, but are devoid of mitochondrial regulators of apoptosis. Cell Death Diff., 8: 1197-206.

Bergamaschi D, et al. 2003. iASPP oncoprotein is a key inhibitor of p53 conserved from worm to human. Nature Genet., 33: 162-7.

Bergeron L, et al. 1998. Defects in regulation of apoptosis in caspase-2-deficient mice. Genes Dev., 12: 1304-1314.

Bergsmedh A, Szeles A, Henriksson M, Bratt A, Folkman MJ, Spetz AL, Holmgren L. 2001. Horizontal transfer of oncogenes by uptake of apoptotic bodies. Proc Natl Acad Sci U S A, 98(11): 6407-11.

Bian F, Nath R, Sobocinski G, Booher RN, Lipinski WJ, Callahan MJ, Pack A, Wang KKW, & Walker LC. 2002. Axonopathy, tau abnormalities, and dyskinesia , but no neurofibrillary tangles in p25-transgenic mice. J. Comp. Neurol., 446: 257-266.

Bibb JA, et al. 1999. Phosphorylation of DARPP32 by Cdk5 modulates dopamine signaling in neurons. Nature, 402: 669-671.

Bibb JA, et al. 2001. Effects of chronic exposure to cocaine are regulated by the neuronal protein Cdk5. Nature, 410: 376-380.

Bilsland J, Harper S. 2002. Caspases and neuroprotection. Curr Opin Investig Drugs, 3(12):1745-52.

Blom WM, de Bont HJ, Meijerman I, Mulder GJ, Nagelkerke JF. 1999. Prevention of cycloheximide-induced apoptosis in hepatocytes by adenosine and by caspase inhibitors. Biochem. Pharmacol., 58(12): 1891-8.

Blomgren K, Zhu C, Wang X, Karlsson J, Leverin A-L, Bahr BA, Mallard C, and Hagberg H. 2001. Synergistic activation of caspase-3 by m-calpain after neonatal hypoxia-ischemia. J. Biol. Chem., 276(13): 10191-10198.

Boise LH, Collins CM. 2001. Salmonella-induced cell death: apoptosis, necrosis or programmed cell death? Trends Microbiol., 9(2):64-7.

Borkhardt A. 2002. Blocking oncogenes in malignant cells by RNA interference-new hope for a highly specific cancer treatment? Cancer Cell, 2(3): 167.

Bowen ID, Mullarkey K, Morgan SM. 1996. Programmed cell death during metamorphosis in the blow-fly Calliphora vomitoria. Microsc Res Tech., 34(3):202-17.

Boxem M, Srinivasan DG, van den Heuvel S. 1999. The Caenorhabditis elegans gene ncc-1 encodes a cdc2-related kinase required for M phase in meiotic and mitotic cell divisions, but not for S phase. Development, 126(10): 2227-39.

Bradford. 1970. Metabolic response of synaptosomes to electrical stimulation: release of amino acids. Brain Res. 19: 239-247.

Brana C, Benham C, Sundstrom L. A method for characterising cell death in vitro by combining propidium iodide staining with immunohistochemistry. Brain Res. Brain Res. Protoc., 10(2): 109-14.

Brancolini C, Benedetti M and Schneider C. 1995. Microfilament reorganization during apoptosis: the role of Gas2, a possible substrate for ICE-like Proteases. EMBO J. 14: 5179-5190.

Bredesen DE. 1995. Neural apoptosis. Ann. Neurol., 38(6): 839-851.

Bu B, Li J, Davies P, & Vincent I. 2002. Deregulation of Cdk5, hyperphosphorylation, and cytoskeletal pathology in the Niemann-Pick type C murine model. J. Neurosci., 22(15): 6515-6525.

Bulavin DV, Amundson SA, Fornace AJ. 2002. p38 and Chk1 kinases: different conductors for the G(2)/M checkpoint symphony. Curr. Opin. Genet. Dev., 12(1): 92-7.

Burns TF, Bernhard EJ, & El-Deiry WS. 2001. Tissue specific expression of p53 target genes suggests a key role for KILLER/DR5 in p53-dependent apoptosis in vivo. Oncogene, 20(34): 4601-4612.

Bursch W, Kienzl H, Ellinger A, Török L, Walker R, Sikorska M, Pandey S, & Schulte-Hermann R. 1996. Active cell death induced by antiestrogens tamoxifen and ICI 164384 in human mammary carcinoma cells (MCF-7) in culture: the role of autophagy. Carcinogenesis, 17: 1595-1607.

Busser J, Geldmacher DS, and Herrup K. 1998. Ectopic cell cycle proteins predict the sites of neuronal cell death in Alzheimer's disease brain. *J. Neurosci.*, 18: 2801-2807.

Buttyan R, Zakcri Z, Lockshin RA, and Wolgcmuth DJ. 1988. Cascadc induction of c-fos, c-myc and heat shock-70K transcripts during regression of the rat ventral prostate gland. Mol. Endocrinology, 2: 650-657.

Cai XH, Tomizawa K, Tang D, Lu YF, Moriwaki A, Tokuda M, Nagahata S, Hatase O and Matsui H. 1997. Changes in the expression of novel Cdk5 activator messenger RNA (p39nck5ai mRNA) during rat brain development. Neurosci. Res., 28: 355-360.

Cao G, Luo Y, Nagayama T, Pei W, Stetler RA, Graham SH, & Chen J. 2002. Cloning and characterization of rat caspase-9: implications for a role in mediating caspase-3 activation and hippocampal cell death after transient cerebral ischemia. J. Cereb. Blood Flow Metab., 22(5): 534-546.

Cecconi F, et al. 1998. Apaf1 (CED-4 homolog) regulates programmed cell death in mammalian development. Cell, 94: 727-737.

Chae T, Kwon YT, Bronson R, Dikes P, Li E and Tsai LH. 1997. Mice lacking p35, a neuronal specific activator of Cdk5, display cortical lamination defects, seizures, and adult lethality. Neuron, 18: 29-42.

Chan SL, and Mattson MP. 1999. Caspase and calpain substrates: roles in synaptic plasticity and cell death. J. Neurosci. Res., 58: 167-190.

Chanoine C, Hardy S. 2003. Xenopus muscle development: From primary to secondary myogenesis. Dev. Dyn., 226(1): 12-23.

Chao C, Saito S, Kang J, Anderson CW, Appella E, Xu Y. 2000. p53 transcriptional activity is essential for p53-dependent apoptosis following DNA damage. EMBO J., 19(18): 4967-75.

Chautan M, Chazal G, Cecconi F, Gruss P, & Golstein P. 1999. Interdigital cell death can occur through a necrotic and caspase-independent pathway. Curr. Biol., 9: 967-970.

Chehab NH, Malikzay A, Appel M, Halazonetis TD. 2000. Chk2/hCds1 functions as a DNA damage checkpoint in G1 by stabilizing p53. Genes Dev., 14: 278-8.

Chen F, Rao J, & Studzinski GP. 2000. Specific association of increased cyclin-dependent kinase 5 expression with monocytic lineage of differentiation of human leukemia HL60 cells. J. Leukoc. Biol., 67: 559-566.

Chen F, & Studzinski GP. 2001. Expression of the neuronal cyclin-dependent kinase 5 activator p35Nck5a in human monocytic cells is associated with differentiation. Blood, 97(12): 3763-3767.

Chen J, et al. 2000. Induction of cyclin-dependent kinase 5 in the hippocampus by chronic electroconvulsive seizures: role of δFosB. J. Neurosci., 20: 8965-8971.

Cheng K, Li Z, Fu WY, Wang JH, Fu AKY, & Ip NY. 2002. Pctaire1 interacts with p35 and is a novel substrate for Cdk5/p35. J. Biol. Chem., 277(35): 31988-31993.

Chernova OB, Chernov MV, Agarwal ML, Taylor WR, & Stark GR. 1995. The role of p53: Regulating genomic stability when DNA and RNA synthesis are inhibited. Trends Bioch. Sci., 20: 431-434.

Choi YJ, Park JW, Woo JH, Kim YH, Lee SH, Lee JM, Kwon TK. 2002. Failure to activate caspase 3 in phorbol ester-resistant leukemia cells is associated with resistance to apoptotic cell death. Cancer Lett., 182(2): 183-91.

Clarke PGH. 1990. Developmental cell death: morphological diversity and multiple mechanisms. Anat. Embryol., 181: 195-213.

Clarke PGH, & Clarke S. 1996. Nineteenth centurial research on naturally occurring cell death and related phenomena. Anat. Embryol., 193: 81-99.

Cleveland DW. 1999. From Charcot to SOD1: mechanisms of selective motor neuron death in ALS. Neuron, 24: 515-520.

Colussi P, Kumar S. 1999. Targeted disruption of caspase genes in mice: what they tell us about the functions of individual caspases in apoptosis. Immun. Cell Biology, 77: 58-63.

Cong J, Goll DE, Peterson AM, and Kapprell HP. 1989. The role of autolysis in activity of the Ca^{2+} dependent proteinases (μ-calpain and m-calpain). J. Biol. Chem., 264: 10096-10103.

Cory S, Adams JM. 2002. The Bcl2 family: regulators of the cellular life-or-death switch. Nat. Rev. Cancer, 2(9): 647-56.

Costanzo A, et al. 2002. DNA damage-dependent acetylation dictates the selective activation of apoptotic target genes. Mol. Cell, 9: 175-86.

Coucouvanis EC, Martin GR, Nadeau JA. 1995. Genetic approaches for studying programmed cell death during development of the laboratory mouse. Methods Cell Biol., 46: 387-440.

Dai JD, Gilbert LI. 1999. An in vitro analysis of ecdysteroid-elicited cell death in the prothoracic gland of Manduca sexta. Cell Tissue Res., 297(2):319-27.

Dalton SR, Wiegert RL, Baldwin CR, Kassel KM, Casey CA. 2003. Impaired receptor-mediated endocytosis by the asialoglycoprotein receptor in ethanol-fed mice: implications for studying the role of this receptor in alcoholic apoptosis. Biochem. Pharmacol., 65(4): 535-43.

Davis PK, Dudek SM, and Johnson GV. 1997. Select alterations in protein kinases and phosphatases during apoptosis of differentiated PC12 cells. *J. Neurochem.*, 68: 2338-2347.

De Laurenzi, et al. 1998. Two new p73 splice variants, γ and δ, with different transcriptional activity. J. Exp. Med., 188: 1763-8.

De Laurenzi, et al. 2000a. Induction of neuronal differentiation by p73 in a neuroblastoma cell line. J. Biol. Chem., 275: 15226-31.

De Laurenzi, et al. 2000b. p63 and p73 transactivate differentiation gene promoters in human keratinocytes. Biochem. Biophys. Res. Commun., 273: 342-6.

de Torres C, Munell F, Roig M, Reventos J, Macaya A. 2002. Naturally occurring cell death during postnatal development of rat skeletal muscle. Muscle Nerve, 26(6):777-83.

Denecker G, Vercammen D, Declercq W, & Vandenabeele P. 2001. Apoptotic and necrotic cell death induced by death domain receptors. Cell. Mol. Life Sci., 58: 356-370.

Dennert G. 2002. Elimination of virus-specific cytotoxic T cells in the liver. Crit. Rev. Immunol., 22(1): 1-11.

DePetrillo PB. 1997. Calcium-activated neutral protease activity is decreased in PC12 cells after ethanol exposure. J. Neurochem., 68: 1863-9.

Dhavan R, Tsai LH. 2001. A decade of CDK5. Nat Rev Mol Cell Biol., 2(10): 749-59.

Dobashi Y, Shoji M, Kitagawa M, Noguchi T, Kameya T. 2000. Simultaneous suppression of cdc2 and cdk2 activities induces neuronal differentiation of PC12 cells. J. Biol. Chem., 275(17):12572-80.

Donehower L, Harvey M, Slagle B, McArthur M, Montgomery C, Butel J, Bradley A. 1992. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature, 356: 215-221.

Eedele M, Pierantoni GM, Berlingieri MT, Battista S, Baldassarre G, Munshi N, Dentice M, Thanos D, Santoro M, Viglietto G, & Fusco A. 2001. Overexpression of proteins HMGA1 induces cell cycle deregulation and apoptosis in normal rat thyroid cells. Cancer Res., 61(11): 4583-4590.

Ekert RG, Silke J, Hawkins CJ, Verhagen AM, Vaux DL. 2001. DIABLO promotes apoptosis by removing MIHA/XIAP form processed caspase 9. J. Cell Biol., 152: 483-90.

Elledge SJ, Spottswood MR. 1991. A new human p34 protein kinase, CDK2, identified by complementation of a cdc28 mutation in Saccharomyces cerevisiae, is a homolog of Xenopus Eg1. EMBO J., 10(9): 2653-9.

Fadeel B, Orrenius S, Henter H. 2001. Familial hemophagocytic lymphohistiocytosis: too little cell death can seriously damage your health. Leuk. Lymphoma, 42(1-2): 13-20.

Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, & Henson PM. 1998. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-β, PGE2, and PAF. J. Clin. Invest., 101: 890-8.

Fahrbach SE, Choi MK, Truman JW. 1994. Inhibitory effects of actinomycin D and cycloheximide on neuronal death in adult Manduca sexta. J Neurobiol., 25(1):59-69.

Fedele M, Pierantoni GM, Berlingieri MT, Battista S, Baldassarre G, Munshi N, Dentice M, Thanos D, Santoro M, Viglietto G, Fusco A. 2001. Overexpression of proteins HMGA1 induces cell cycle deregulation and apoptosis in normal rat thyroid cells. Cancer Res., 61(11): 4583-90.

Fei P, Bernhard EJ, El-Deiry WS. 2002. Tissue-specific induction of p53 targets in vivo. Cancer Res., 62(24): 7316-27.

Finlay CA, Hinds PW, & Levine AJ. 1989. The p53 proto-oncogene can act as a suppressor of transformation. Cell, 57: 1083-1093.

Fletcher AI, Shuang R, Giovannucci DR, Zhang L and Bittner MA. 1999. Regulation of exocytosis by cyclin-dependent kinase 5 via phosphorylation of Munc18. J. Biol. Chem. 274: 4027-4035.

Flores ER, et al. 2002. p63 and p73 are required for p53-dependent apoptosis in response to DNA damage. Nature, 416: 560-4.

Floyd SR, et al. 2001. Amphiphysin 1 binds the cyclin-dependent kinase 5 (Cdk5) regulatory subunit p35 and is phosphorylated by Cdk5 and Cdc2. J. Biol. Chem., 276: 8104-8110.

Foghsgaard, L. *et al.* 2001. Cathepsin B acts as a dominant execution protease in tumor cell apoptosis induced by tumor necrosis factor. *J. Cell Biol.* 153: 999–1009.

Fox H. 1973. Ultrastructure of tail degeneration in Rana temporaria larvae. Folia Morphol., (Prague) 21: 109-12.

Francis BM, Rogers JM, Sulik KK, Alles AJ, Elstein KH, Zuker RM, Massaro EJ, Rosen MB and Chernoff N (1990) Cyclophosphamide teratogenesis: evidence for compensatory response to induced cellular toxicity. Teratology 42: 473-482

Friedlander RM, et al. 1997. Expression of a dominant negative mutant of interleulin-1 beta converting enzyme in transgenic mice prevents neuronal cell death induced by trophic factor withdrawal and ischemic brain injury. J. Exp. Med., 185: 933-940.

Fu AK, et al. 2001. Cdk5 is involved in neuregulin-induced AChR expression at the neuromuscular junction. Nature Neurosci., 4: 374-381.

Fu WY, Wang JH, & Ip NY. 2002. Expression of Cdk5 and its activators in NT2 cells during neuronal differentiation. J. Neurochem., 81: 646-654.

Fukuda H. 2001. Programmed cell death of tracheary elements as a paradigm in plants. Plant Mol. Biol., 44: 229-235.

Fujimoto K, Iwakiri R, Wu B, Fujise T, Tsunada S, Ootani A. 2002. Homeostasis in the small intestinal mucosa balanced between cell proliferation and apoptosis is regulated partly by the central nervous system. J. Gastroenterol., Suppl. 14: 139-44.

Gadducci A, Cosio S, Muraca S, Genazzani AR. 2002. Molecular mechanisms of apoptosis and chemosensitivity to platinum and paclitaxel in ovarian cancer: biological data and clinical implications. Eur J Gynaecol Oncol., 23(5):390-6.

Galati D, Bocchino M, Paiardini M, Cervasi B, Silvestri G, Piedimonte G. 2002. Cell cycle dysregulation during HIV infection: perspectives of a target based therapy. Curr. Drug Targets Immune. Endocr. Metabol. Disord., 2(1):53-61.

Gao CY, Zakeri Z, Zhu Y, He H, and Zelenka PS. 1997. Expression of Cdk5, p35, and Cdk5 associated kinase activity in the developing rat lens. Dev. Genet., 20: 267-275.

Gao CY, and Zelenka PS. 1995. Induction of cyclin B and H1 kinase activity in apoptotic PC12 cells. *Exp. Cell Res.*, 219: 612-618.

Genini D, Sheeter D, Rought S, Zaunders JJ, Susin SA, Kroemer G, Richman DD, Carson DA, Corbeil J, & Leoni LM. 2001. HIV induces lymphocyte apoptosis by a p53-initiated, mitochondrial-mediated mechanism. FASEB J., 15(1): 5-6.

Gercel-Taylor C, O'Connor SM, Lam GK, Taylor DD. 2002. Shed membrane fragment modulation of CD3-zeta during pregnancy: link with induction of apoptosis. J. Reprod. Immunol., 56(1-2): 29-44.

Gervasi C, Szaro BG. 1995. The Xenopus laevis homologue to the neuronal cyclin-dependent kinase (cdk5) is expressed in embryos by gastrulation. Brain Res. Mol. Brain Res., 33: 192-200.

Ghahremani MH, et al. 2002. Interaction of the c-Jun/JNK pathway and cyclin-dependent kinases in death of embryonic cortical neurons evoked by DNA damage. J. Biol. Chem., 277(38): 35586-96.

Giardina SF, Beart PM. 2002. Kainate receptor-mediated apoptosis in primary cultures of cerebellar granule cells is attenuated by mitogen-activated protein kinase and cycle-dependent kinase inhibitors. Br. J. Pharmacol., 135(7): 1733-42.

Glassford A, Lee JE, Xu L, Giffard RG. 2002. Caspase inhibitors reduce the apoptotic but not necrotic component of kainate injury in primary murine cortical neuronal cultures. Neurol. Res., 24(8): 796-800.

Glucksmann A. 1951. Cell deaths in normal vertebrate ontogeny. Biol. Rev. Cambridge Philos. Soc., 26: 59.

Gong J, Li X, Darzynkiewicz Z. 1993. Different patterns of apoptosis of HL-60 cells induced by cycloheximide and camptothecin. J. Cell Physiol., 157(2): 263-70.

Gordon N. 1995. Apoptosis (programmed cell death) and other reasons for elimination of neurons and axons. Brain Dev., 17: 73-7.

Goyal L, McCall K, Agapite J, Hartwieg E, & Steller H. 2000. Induction of apopotosis by Drosophila reaper, hid, and grim through inhibition of IAP function. EMBO J., 19: 589-597.

Grant P, Sharma P, & Pant HC. 2001. Cyclin-dependent protein kinase 5 (Cdk5) and the regulation of neurofilament metabolism. Eur. J. Biochem., 268: 1534-1546.

Greengard R, Allen PB, & Nairn AC. 1999. Beyond the dopamine receptor: the DARPP-32/protein phosphatase-1 cascade. Neuron, 23: 435-447.

Gu Y, Rosenblatt J, Morgan DO. 1992. Cell cycle regulation of Cdk2 activity by phosphorylation of Thr160 and Tyr15. EMBO J., 11: 3995-4005.

Gupta A, & Tsai LH. 2001. A kinase to dampen the effects of cocaine. Science, 292(5515): 236.

Gupta A, Tsai LH, & Wynshaw-Boris A. 2002. Life is a journey: a genetic look at neocortical development. Nat. Rev. Genet., 3(5): 342-55.

Guicciardi, M.E. *et al.* 2000. Cathepsin B contributes to TNF-α-mediated hepatocyte apoptosis by promoting mitochondrial release of cytochrome *c. J. Clin. Invest.* 106: 1127–1137.

Ha HC, Snyder SH. 1999. Poly(ADP-ribose) polymerase is a mediator of necrotic cell death by ATP depletion. Proc. Natl. Acad. Sci. USA, 96: 13978-82.

Haas AL, Baboshina O, Williams B, Schwartz LM. 1995. Coordinated induction of the ubiquitin conjugation pathway accompanies the developmentally programmed death of insect skeletal muscle. J. Biol. Chem., 270(16): 9407-12.

Haecker G, & Vaux DL. 1994. Viral, worm and radical implications for apoptosis. Trends Biochem. Sci., 19: 99-100.

Hager JH, & Hanahan D. 1999. Tumor cells utilize multiple pathways to down-modulate apoptosis. Lessons from a mouse model of islet cell carcinogenesis. Ann. N Y Acad. Sci., 887: 150-163.

Hakem R, Hakem A, Duncan GS, Henderson JT, Woo M, Soengas MS, Elia A, de la Pompa JL, Kagi D, Khoo W, Potter J, Yoshida R, Kaufman SA, Lowe SW, Penninger JM, Mak TW. 1998. Differential requirement for caspase 9 in apoptotic pathways in vivo. Cell, 94(3): 339-52.

Halterman MW, Miller CC, & Federoff HJ. 1999. Hypoxia-inducible factor-1 alpha mediates hypoxia-induced delayed neuronal death that involves p53. J. Neurosci., 19: 6818-6824.

Han BH, Xu D, Choi J, Han Y, Xanthoudakis S, Roy S, Tam J, Vaillancourt J, Colucci J, Siman R, Giroux A, Robertson GS, Zamboni R, Nicholson DW, & Hotzman DM. 2002. Selective, reversible caspase-3 inhibitor is neuropretective and reveals distinct pathways of cell death after neonatal hypoxic-ischemic brain injury. J. Biol. Chem., 277(33): 30128-30136.

Harada T, Morooka T, Ogawa S, & Nishida E. 2001. ERK induces p35, a neuron-specific activator of Cdk5, through induction of Egr1. Nature Cell Biol., 3: 453-459.

Hardy K, Handyside AH, Winston RM. 1989. The human blastocyst: cell number, death and allocation during late preimplantation development in vitro. Dev., 107: 597-604.

Hashiguchi M, Saito T, Hisanaga S, Hashiguchi T. 2002. Truncation of CDK5 Activator p35 Induces Intensive Phosphorylation of Ser202/Thr205 of Human Tau. J Biol Chem., 277(46): 44525-30.

Hauck L, Hansmann G, Dietz R, & von Harsdorf R. 2002. Inhibition of hypoxia-induced apoptosis by modulation of retinoblastoma protein-dependent signaling in cardiomyocytes. Circ. Res., 91(9): 782-789.

Hayashi F, et al. 2000. Phosphorylation by cyclin-dependent protein kinase 5 of the regulatory subunit of retinal cGMP phosphodiesterase. II. Its role in the turnoff of phosphodiesterase in vivo. J. Biol. Chem., 275: 32958-32965.

Hegde R, Srinivasula SM, Zhang Z, Wassell R, Mukattash R, Cilenti L, DuBois G, Lazebnik Y, Zervos AS, Fernandes-Alnemri T, & Alnemri ES. 2002. Identification of Omi/HtrA2 as a mitochondrial apoptotic serine protease that disrupts IAP-caspase interaction. J. Biol. Chem., 277(1): 432-8.

Heibein JA, Barry M, Motyka B, & Bleackley RC. 1999. Granzyme B-induced loss of mitochondrial inner membrane potential (Kelta Psi m) and cytochrome c release are caspase-dependent. J. Immunol., 163: 4683-4693.

Heintz N. 1993. Cell death and the cell cycle: a relationship between transformation and neurodegeneration? *Trends Biochem. Sci.*, 18: 157-179.

Hellmich MR, Kennison JA, Hampton LL, & Battey JF. 1994. Cloning and characterization of the Drosophila melanogaster Cdk5 homolog. FEBS Lett., 356(2-3): 317-21.

Hellmich MR, Pant HC, Wada E, Battey JF. 1992. Neuronal cdc2-like kinase: a cdc2-related protein kinase with predominantly neuronal expression. Proc Natl Acad Sci U S A, 89(22): 10867-71.

Henchcliffe C and Burke RE. 1997. Increased expression of cyclin-dependent kinase 5 in induced apoptotic neuron death in rat substantia nigra. Neurosci. Lett. 230: 41-44.

Henkart PA. 1996. ICE family proteases: mediators of all apoptotic cell death? Immunity, 4(3): 195-201.

Henmi K, Tsuboi S, Demura T, Fukuda H, Iwabuchi M, Ogawa KI. 2001. A possible role of glutathione and glutathione disulfide in tracheary element differentiation in the cultured mesophyll cells of Zinnia elegans. Plant Cell Physiol., 42(6): 673-6.

Heusel JW, Wesselschmidt RI, Shresta S, Russell JH, & Ley TJ. 1994. Cytotoxic lymphocytes require granzyme B for the rapid induction of DNA fragmentation and apoptosis in allogeneic target cells. Cell, 76: 977-987.

Hickman ES, Moroni MC, Helin K. 2002. The role o fp53 and pRB in apoptosis and cancer. Curr. Opin. Genet. Dev., 12(1): 60-6.

Hoeberichts FA, Woltering EJ. 2002. Multiple mediators of plant programmed cell death: interplay of conserved cell death mechanisms and plant-specific regulators. BioEssays, 25: 47-57.

Hofmann HS, Simm A, Hammer A, Silber RE, & Bartling B. 2002. Expression of inhibitors of apoptosis (IAP) proteins in non-small cell human lung cancer. J. Cancer Res. Clin. Oncol., 128(10): 554-60.

Holler N, Zaru R, Micheau O, Thome M, Attinger A, Valitutti S, Bodmer JL, Schneider P, Seed B, Tschopp J. 2000. Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. Nat. Immunol., 1(6): 489-95.

Horvitz HR, Ellis HM, & Sternberg PW. 1982. Programmed cell death in nematode development. Neurosci. Comment., 1: 56-65.

Huang D, Patrick G, Moffat J, Tsai LH, & Andrews B. 1999. Mammalian Cdk5 is a functional homologue of the budding yeast Pho85 cyclin-dependent kinase. Proc. Natl. Acad. Sci. USA, 96: 14445-14450.

Huang H, Joazeiro CA, Bonfoco E, Kamada S, Leverson JD, Hunter T. 2000. The inhibitor of apoptosis, cIAP2, functions as a ubiquitin-protein ligase and promotes in vitro monoubiquitination of caspases 3 and 7. J. Biol. Chem., 275(35):26661-4.

Huang JB, Kindzelskii AL, Petty HR. 2002. Hexokinase translocation during neutrophil activation, chemotaxis, and phagocytosis: disruption by cytochalasin D, dexamethasone, and indomethacin. Cell Immunol., 218(1-2): 95-106.

Huang RF, Huang SM, Lin BS, Wei JS, & Liu TZ. 2001. Homocysteine thiolactone induces apoptotic DNA damage mediated by increased intracellular hydrogen peroxide and caspase 3 activation in HL-60 cells. Life Sci., 68(25): 2799-811.

Humbert S, Dhavan R, & Tsai LH. 2000a. p39 activates Cdk5 in neurons, and is associated with the actin cytoskeleton. J. Cell Sci., 113: 975-983.

Humbert S, Lanier LM, & Tsai LH. 2000b. Synaptic localization of p39, a neuronal activator of Cdk5. Neuroreport, 11: 2213-6.

Hurle JM, Colombatti A. 1996. Extracellular matrix modifications in the interdigital spaces of the chick embryo leg bud during the formation of ectopic digits. Anat. Embryol. (Berl), 193(4): 355-64.

Iijima K, et al. 2000. Neuron-specific phosphorylation of Alzheimer's β-amyloid precursor protein by cyclin-dependent kinase 5. J. Neurochem., 75: 1085-1091.

Imahori K, Hoshi M, Ishiguro K, Sato K, Takahashi M, Shiurba R, Yamaguchi H, Takashima A, and Uchida T. 1998. Possible of tau protein kinases in pathogenesis of Alzheimer's disease. *Neurobiol. Aging*, 19: S93-S98.

Ino H, Ishizuka T, Chiba T and Tatibana M. 1994. Expression of Cdk5 (PSSALRE kinase), a neural cdc2-related protein kinase, in the mature and developing mouse central and peripheral nervous systems. Brain Res. 661: 196-206.

Ishiguro K, Takamatsu M, Tomizawa K, Omori A, Takahashi M, Arioka M, Uchida T, and Imahori K. 1992. Tau protein kinase I converts normal tau protein into A68-like component of paired helical filaments. J. Biol. Chem., 267: 10897-10901.

Ishiguro K, Kobayashi S, Omori A, Takamatsu M, Yonekura S, Anzai K, Imahori K and Uchida T. 1994. Identification of the 23 kDa subunit of tau protein kinase II as a putative activator of Cdk5 in bovine brain. *FEBS Lett.*, 342: 203-208.

Iwamoto H, Miura T, Okamura T, Shirakawa K, Iwatate M, Kawamura S, Tatsuno H, Ikeda Y, and Matsuzaki M. 1999. Calpain inhibitor-1 reduces infarct size and DNA fragmentation of myocardium in ishcemic/reperfused rat heart. J. Cardiovasc. Pharmacol., 33: 580-586.

Jacobson MD, Weil M, & Raff MC. 1997. Programmed cell death in animal development. Cell, 88: 347-354.

Jeffery WR. 2002. Programmed cell death in the ascidian embryo: modulation by FoxA5 and Manx and roles in the evolution of larval development. Mech. Dev., 118(1-2): 111-24.

Jellinger KA. 2001. Cell death mechanisms in neurodegeneration. J. Cell Mol. Med., 5(1): 1-17.

Jiang X, et al. 2003. Distinct roles of PHAP and prothymosin-α in a djeath regulatory pathway. Science, 299: 223-6.

Jochova J, Quaglino D, Zakeri Z, Woo K, Sikorska M, Weaver V, & Lockshin RA. 1997. Protein synthesis, DNA degradation, and morphological changes during programmed cell death in labial glands of Manduca Sexta. Dev. Genet., 21: 249-257.

Jochova J, Zakeri Z and Lockshin RA. 1997. Early collapse of the cytoskeleton in the programmed cell death of the Drosophila salivary gland. Cell Death Diff., 5: 140-149

Johnson, D.E. 2000. Noncaspase proteases in apoptosis. *Leukemia,* 14: 1695–1703.

Jost CA, Marin MC, Kaelin WG. 1997. p73 is a simian p53-related protein that can induce apoptosis. Nature, 389: 191-4.

Juo P, Kuo CJ, Yuan J, & Blenis J. 1998. Essential requirement for caspase-8/FLICE in the initiation of the Fas-induced apoptotic cascade. Curr. Biol., 8: 1001-1008.

Kagi D, Vignaux F, Ledermann B, Burki K, Kepreatere V, Nagata S, Hengartner H. 1994. Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. Science, 265(5171): 528-530.

Karasavvas N, Erukulla RK, Bittman R, et al. 1996. Stereospecific induction of apoptosis in U937 cells by N-octanoyl-sphingosine stereoisomers and the novel ceramide analog N-octyl-sphingosine. Eur. J. Biochem., 236: 729-737.

Karpinich NO, Tafani M, Rothman RJ, Russo MA, & Farber JL. 2002. The course of etoposide-induced apoptosis from damage to DNA and p53 activation to mitochondrial release of cytochrome C. J. Biol. Chem., 277(19): 16547-16552.

Katner AL, Gootam P, Hoang QB, Gnarra JR, Rayford W. 2002. A recombinant adenovirus expressing p7(Kip1) induces cell cycle arrest and apoptosis in human 786-0 renal carcinoma cells. J. Urol., 168: 766-73.

Kato G, & Maeda S. 1999. Neuron-specific Cdk5 kinase is responsible for mitosis-independent phosphorylation of c-Src at Ser75 in human Y79 retinoblastoma cells. J. Biochem., 126: 957-961.

Kazzaz JA, Horowitz S, Xu J, Khullar P, Niederman MS, Fein AM, Zakeri Z, Lin L, and Rhodes GC. 1999. Differential patterns of apoptosis in resolving and nonresolving bacterial pneumonia. *American Journal of Respiratory and Critical Care Medicine*, 161: 2043-2050.

Kerokoski P, Suuronen T, Salminen A, Soininen Hikka, & Pirttilä T. 2002. Cleavage of the cyclin-dependent kinase 5 activator p35 to p25 does not induce tau hyperphophorylation. Biochem. Biophysic. Res. Commun., 298: 693-698.

Kerr JF, Wyllie AH, & Currie AR. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br. J. Cancer, 26: 239-257.

Kesavapany S, et al. 2001. p35/Cdk5 binds and phosphorylates β-catenin and regulates β-catenin/presenilin-1 interaction. Eur. J. Neurosci., 13: 241-7.

Kim SO, Katz S, Pelech SL. 1998. Expression of second messenger- and cyclin-dependent protein kinases during postnatal development of rat heart. J. Cell Biochem., 69(4): 506-21.

Kitanaka C, & Kuchino Y. 1999. Caspase-independent programmed cell death with necrotic morphology. Cell Death Differ., 6: 508-515.

Ko J, et al. 2001. p35 and p39 are essential for Cdk5 function during neurodevelopment. J. Neurosci., 21: 6758-6771.

Koff A, Cross F, Fisher A, Schumacher J, Leguellec K, Philippe M, Roberts JM. 1991. Human cyclin E, a new cyclin that interacts with two members of the CDC2 gene family. Cell, 66(6): 1217-28.

Kogel D, Reimertz C, Dussmann H, Mech P, Scheidtmann KH, Prehn JH. 2003. The death associated protein (DAP) kinase homologue Dlk/ZIP kinase induces p19ARF- and p53-independent apoptosis. Eur. J. Cancer, 39(2): 249-56.

Kondo S, Barnett GH, Hara H, Morimura T, & Takeuchi J. 1995. MDM2 protein confers the resistance of a human glioblastoma cell line to cisplatin-induced apoptosis. Oncogene, 10(10): 2001-2006.

Kondratyev A, Selby D, & Gale K. 2002. Status epilepticus leads to the degradation of the endogenous inhibitor of caspase-activated DNase in rats. Neurosci. Lett., 319(3): 145-8.

Kuida K, et al. 1998. Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. Cell, 94: 325-337.

Kuida K, Zheng TS, Na S, Kuan C, Yang D, Karasuyama H, Rakic R, & Flavell RA. 1996. Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. Nature, 384: 368-72.

Kung AL, Zetterberg A, Sherwood SW, Schimke RT. 1990. Cytotoxic effects of cell cycle phase specific agents: result of cell cycle perturbation. Cancer Res., 50(22): 7307-17.

Kuriyama H, Fukuda H. 2002. Developmental programmed cell death in plants. Curr. Opin. Plant Biol., 5(6): 568-73.

Kusakawa GI, Saito T, Onuki R, Ishiguro K, Kishomoto T and Hisanaga S. 2000. Calpain-dependent proteolytic cleavage of the p35 cyclin-dependent kinase 5 activator to p25. J. Biol. Chem. 275: 17166-17172

Kuwako K, Nishimura I, Uetsuki T, Saido TC, & Yoshikawa K. 2002. Activation of calpain in cultured neurons overexpressing Alzheimer amyloid precursor protein. Brain Res. Mol. Brain Res., 107(2): 166-175.

Kuwana T, Mackey MR, Perkins G, Ellisman MH, Latterich M, Schneiter R, Green DR, Newmeyer DD. 2002. Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. Cell, 111(3): 331-42.

Kwon YT, Gupta A, Zhou Y, Nikolic M and Tsai LH. 2000. Regulation of N-cadherin-mediated adhesion by the p35/cdk5 kinase. Curr. Biol. 10: 363-372.

Kyprianou N, Bruckheimer EM, & Guo Y. 2000. Cell proliferation and apoptosis in prostate cancer: significance in disease progression and therapy. Histol. Histopathol., 15(4): 1211-1223.

Laemmli EK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

Lane DP, & Crawford LV. 1979. T antigen is bound to a host protein in SV40-transformed cells. Nature, 278: 261-263.

Lang D, Dohle F, Terstesse M, Bangen P, August C, Pauels HG, Heidenreich S. 2002. Down-regulation of monocyte apoptosis by phagocytosis of platelets: involvement of a caspase-9, caspase-3, and heat shock protein 70-dependent pathway. J. Immunol., 168(12): 6152-8.

Lazaro JB, Kitzmann M, Poul MA, Vandromme M and Lamb NJC. 1997. Cyclin dependent kinase 5, cdk5, is a positive regulator of myogenesis in mouse C2 cells. J. Cell Sci., 110: 1251-1260.

Lee CY, Cooksey BA, Baehrecke EH. 2002. Steroid regulation of midgut cell death during Drosophila development. Dev Biol., 250(1):101-11.

Lee KY, Helbing CC, Choi KS, Johnston RN, & Wang JH. 1997. Neuronal Cdc2-like kinase (Nclk) binds and phosphorylates the retinoblastoma protein. J. Biol. Chem., 272: 5622-5626.

Lee MK and Cleveland DW. 1996. Neuronal intermediate filaments. Annu. Rev. Neurosci. 19: 187-217.

Lee M, Kwon KT, Li M, Peng J, Friedlander RM and Tsai LH. 2000. Neurotoxicity induces cleavage of p35 to p25 by calpain. Nature, 405: 360-364.

Lefevre K, Clarke PG, Danthe EE, Castagne V. 2002. Involvement of cyclin-dependent kinases in axotomy-induced retinal ganglion cell death. J. Comp. Neurol., 447(1): 72-81.

Leist M, Gantner F, Bohlinger I, Tiegs G, Germann PG, & Wendel A. 1995. Tumor necrosis factor-induced hepatocyte apoptosis precedes liver failure in experimental murine shock models. Am. J. Pathol., 146: 1220-1234.

Leist M, Gantner F, Kunstle G, Bohlinger I, Tiegs G, & Bluethmann H. 1996. The 55-kD tumor necrosis factor receptor and CD95 independently signal murine hepatocyte apoptosis and subsequent liver failure. Mol. Med., 2: 109-124.

Leist M, Jaattela M. 2001. Four deaths and a funeral: from caspases to alternative mechanisms. Mol. Cell Biol., 2: 589-98.

Lew J, Huang QQ, Winkfein RJ, Aebersold R, Hunt T and Wang JH. 1994. A brain-specific activator of cyclin-dependent kinase 5. Nature, 371:423-6.

Lew J, Wang JH. 1995. Neuronal cdc2-like kinase. Trends Biochem Sci., 20(1): 33-7.

Lew J, Winkffin RJ, Paudel HK and Wang JH. 1992. Purification and characterization of a novel proline-directed protein kinase from bovine brain. J. Biol. Chem., 267: 25922-25926.

Li BS, Zhang L, Gu J, Amin ND, & Pant HC. 2000. Integrin alpha(1) beta(1)-mediated activation of cyclin-dependent kinase 5 activity is involved in neurite outgrowth and human neurofilament protein H Lys-Ser-Pro tail domain phosphorylation. J. Neurosci., 20: 6055-6062.

Li CQ, Trudel LJ, & Wogan GN. 2002. Nitric oxide-induced genotoxicity, mitochondrial damage, and apoptosis in human lymphoblastoid cells expression wild-type and mutant p53. Proc. Natl. Acad. Sci. USA, 99(16): 10364-10369.

Li PA, Howlett W, He QP, Miyashita H, Siddiqui M, and Shuaib A. 1998. Post-ischemic treatment with calpain inhibitor MDL-28170 ameliorates brain damage in a gerbil model of global ischemia. Neurosci. Lett., 247: 17-20.

Li H, and Yuan J. 1999. Deciphering the pathways of life and death. Current Opinion in Cell Biology, 11: 261-266.

Li Y, Chopp M, Jiang N, Zhang ZG, & Zaloga C. 1995. Induction of DNA fragmentation after 10 to 120 minutes of focal cerebral ischemia in rats. Stroke, 26: 1252-8.

Li Z, Hromchak R, Bloch A. 1997. Differential expression of proteins regulating cell cycle progression in growth vs. differentiation. Biochim. Biophys. Acta, 1356(2): 149-59.

Lin Y, Ma W, Benchimol S. 2000. Pidd, a new death-domain-containing protein, is induced by p53 and promotes apoptosis. Nat. Genet., 26: 122-7.

Linzer DIH, & Levine AJ. 1979. Characterization of a 54K Dalton cellular SV40 tumor antigen in SV40 transformed cells. Cell, 17: 43-52.

Little SA, Mirkes PE. 2002. Teratogen-induced activation of caspase-9 and the mitochondrial apoptotic pathway in early postimplantation mouse embryos. Toxicol. Appl. Pharmacol., 181(2): 142-51.

Liu D, Matzuk MM, Sung WK, Guo Q, Wang P, & Wolgemuth DJ. 1998. Cyclin A1 is required for meiosis in the male mouse. Nature genetics, 20: 377-380.

Liu Q, Hilsenbeck S, Gazitt Y. 2003. Arsenic trioxide-induced apoptosis in myeloma cells: p53-dependent G1 or G2/M cell cycle arrest, activation of caspase 8 or caspase 9 and synergy with APO2/TRAIL. Blood 2003 Jan 16.

Liu X, Kim CN, Yang J, Jemmerson R, & Wang X. 1996. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell, 86: 147-157.

Lockshin RA, Williams CM. 1965. Programmed cell death: Neural control of the breakdown of the intersegmental muscles. J. Insect. Physiol., 11: 605-10.

Loo DT, et al. 1993. Apoptosis is induced by β-amyloid in cultured central nervous system neurons. Proc. Natl. Acad. Sci. USA, 90: 7951-7955.

Lorenzo A, et al. 2000. Amyloid- β interacts with the amyloid precursor protein: a potential toxic mechanism in Alzheimer's disease. Nature Neurosci., 3: 460-464.

Los M, Stroh C, Janicke RU, Engels IH, & Schulze-Osthoff K. 2001. Caspases: more than just killers? Trends Immunol., 22(1):31-4.

Lowe SW, & Ruley HE. 1993. Stabilization of the p53 tumor suppressor is induced by adenovirus 5 E1A and accompanies apoptosis. Genes Dev., 7(4): 535-545.

Lu M, Kitson RP, Xue Y, Goldfarb RH. 2003. Activation of multiple caspases and modification of cell surface fas (CD95) in proteasome inhibitor-induced apoptosis of rat natural killer cells. J Cell Biochem., 88(3):482-492.

Lu S, Hoestje SM, Choo E, Epner DE. 2003. Induction of caspase-dependent and -independent apoptosis in response to methionine restriction. Int J Oncol., 22(2):415-20.

Iyer R, Ding L, Batchu RB, Naugler S, Shammas MA, Munshi NC. 2003. Antisense p53 transduction leads to overexpression of bcl-2 and dexamethasone resistance in multiple myeloma. Leuk Res., 27(1): 73-8.

Madesh M, Antonsson B, Srinivasula SM, Alnemri ES, & Hajnozzky G. 2002. Rapid kinetics of tBid-induced cytochrome c and Smac/DIABLO release and mitochondrial depolarization. J. Biol. Chem., 277(7): 5651-9.

Mandelkow EM and Mandelkow E. 1998. Tau in Alzheimer's disease. Trends Cell Biol. 8: 425-427.

Mantell LL, Shaffer TH, Horowitz S, Foust III R, Wolfson MR, Cox C, Khullar P, Zakeri Z, Lin L, Kazzaz JA, Palaia T, Scott W, and Davis JM. 2002. Distinct patterns

of apoptosis in the lung during liquid ventilation compared to gas ventilation. Am J Physiol Lung Cellular and Molecular Physiology, 283: L31-L41.

Marsden VS, O'Connor L, O'Reilly LA, Silke J, Metcalf D, Ekert PG, Huang DC, Cecconi F, Kuida K, Tomaselli KJ, Roy S, Nicholson DW, Vaux DL, Bouillet P, Adams JM, Strasser A. 2002. Apoptosis initiated by Bcl-2-regulated caspase activation independently of the cytochrome c/Apaf-1/caspase-9 apoptosome. Nature, 419(6907): 634-7.

Marsden VS, Strasser A. 2003. Control of Apoptosis in the Immune System: Bcl-2, BH3-Only Proteins and More. Annu. Rev. Immunol., 21: 71-105.

Martin LJ, et al. 1998. Neurodegeneration in excitotoxicity, global cerebral ischemia, and target deprivation: a perspective on the contributions of apoptosis and necrosis. Brain Res. Bull., 46: 281-309.

Martin SJ, & Green DR. 1995. Protease activation during apoptosis: death by a thousand cuts? Cell, 82(3): 349-52.

Martins LM, Iaccarino I, Tenev T, Gschmeissner S, Totty NF, Lemoine NR, Savopoulos J, Gray CW, Creasy CL, Dingwall C, Downward J. 2001. The serine protease Omi/HtrA2 regulates apoptosis by binding XIAP through a reaper-like motif. J. Biol. Chem., 277(1): 439-44.

Mathiase IS, Jaattela M. 2002. Triggering caspase-independent cell death to combat cancer. Trends Mol. Med., 8(5): 212-20.

Matsubara M, et al. 1996. Site-specific phosphorylation of synapsin 1 by mitogen-activated protein kinase and Cdk5 and its effects on physiological functions. J. Biol. Chem., 271: 21108-21113.

Matsuura I, & Wang JH. 1996. Demonstration of cyclin-dependent kinase inhibitory serine/threonine kinase in bovine thymus. J. Biol. Chem., 271: 5443-5450.

Mattson MP, Tomaselli KJ, & Rydel RE. 1993. Calcium-destablizing and neurodegenerative effect of aggregate beta-amyloid peptide are attenuated by basic FGF. Brain Res., 621: 35-49.

McIlroy D, Sakahira H, Talanian RV, & Nagata S. 1999. Involvement of caspase-3-activated DNase in internucleosomal DNA cleavage induced by diverse apoptotic stimuli. Oncogene, 18(31): 4401-8.

Meijer L, Borgne A, Mulner O, Chong JP, Blow JJ, Inagaki N, Inagaki M, Delcros JG, Moulinoux JP. 1997. Biochemical and cellular effects of roscovitine, a potent and selective inhibitor of the cyclin-dependent kinases cdc2, cdk2 and cdk5. Eur. J. Biochem., 243(1-2): 527-36.

Meikrantz W, Gisselbrecht S, Tam SW, Schlegel R. 1994. Activation of cyclin A-dependent protein kinases during apoptosis. Proc Natl Acad Sci U S A, 91(9): 3754-8.

Meikrantz W, Schlegel R. 1996. Suppression of apoptosis by dominant negative mutants of cyclin-dependent protein kinases. J. Biol. Chem., 271(17): 10205-9.

Melino G, De Laurenzi V, Vousden KH. 2002. p73: Friend or foe in tumorigenesis. Nat. Rev. Cancer, 2(8): 605-15.

Meyerson M, Enders GH, Wu CL, Su LK, Gorka C, Nelson C, Harlow E and Tsai LH. 1992. A family of human cdc2-related protein kinases. EMBO J. 11: 2909-2917.

Michael D, Oren M. 2002. The p53 and Mdm2 families in cancer. Curr. Opin. Genet. Dev., 12(1): 53-9.

Michel G, Mercken M, Murayama M, Noguchi K, Ishiguro K, Imahori K, and Takashima A. 1998. Characterization of tau phosphorylation in glycogen synthase kinase-3beta and cyclin dependent kinase-5 activator (p23) transfected cells. *Biochim, Biophys. Acta.*, 1380: 177-182.

Mirkes PE. 1985. Cyclophosphamide teratogenesis: A review. Teratology 32: 259-266.

Miyashita T, Krajewski S, Krajewska M, Wang HG, Lin HK, Liebermann DA, Hoffman B, Reed JC. 1994. Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. Oncogene, 9: 1799-1805.

Moallem SA and Hales BF. 1995. Induction of apoptosis and cathepsin D in limbs exposed in vitro to an activated analog of cyclophosphamide. Teratology 52: 3-14.

Mohamed YH, Amemiya T. 2003. Apoptosis and lens vesicle development. Eur. J. Ophthalmol., 13(1): 1-10.

Moretti A, Weig HJ, Ott T, Seyfarth M, Holthoff HP, Grewe D, Gillitzer A, Bott-Flugel L, Schomig A, Ungerer M, Laugwitz KL. 2002. Essential myosin light chain as a target for caspase-3 in failing myocardium. Proc Natl Acad Sci U S A, 99(18):11860-5.

Moroni MC, Hickman ES, Caprara G, Denchi E, Colli E, Cecconi F, Muller H, Helin K. 2001. Apaf1 is a transcriptional target for E2F and p53. Nat. Cell Biol., 3: 552-8.

Morris EJ, Keramaris E, Rideout HJ, Slack RS, Dyson NJ, Stefanis L, & Park DS. 2001. Cyclin-dependent kinases and p53 pathways are activated independently and mediate Bax activation in neurons after DNA damage. J. Neurosci., 21: 5017-5026.

Motoyama N, Wang FP, Roth KA, Sawa H, Nakayama K, Negishi I, Senju S, Zhang Q, Fujii S, et al. 1995. Massive cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice. Science, 267: 1506-1510.

Musa FR, et al. 1998. Expression of cyclin-dependent kinase 5 and associated cyclins in Leydig and Sertoli cells of the testis. J. Androl., 19: 657-666.

Nagata T, Kishi H, Liu QL, Matsuda T, Imanaka T, Tsukada K, Kang D, & Muraguchi A. 2002. The regulation of DNase activates in subcellular compartments of activated thymocytes. Immun., 105(4): 399-406.

Nahle Z, Polakoff J, Davuluri RV, McCurrach ME, Jacobson MD, Narita M, Zhang MQ, Lazebnik Y, Bar-Sagi D, Lowe SW. 2002. Direct coupling of the cell cycle and cell death machinery by E2F. Nat. Cell Biol., 4(11): 859-64.

Nakano K, Vousden K. 2001. PUMA, a novel proapoptotic gene, is induced by p53. Mol Cell, 7: 683-94.

Nath R, Davis M, Probert AW, Kupina NC, Ren X, Schielke GP, and Wang KKW. 2000. Processing of cdk5 activator p35 to its truncated form (p25) by calpain in acutely injured neuronal cells. Biochem. Biophys. Res. Commun., 274: 16-21.

Nath R et al. 1996a. Non-erythroid alpha-spectrin breakdown by calpain and interleukin 1β-converting-enzyme-like protease(s) in apoptotic cells, contributory roles of both protease families in neuronal apoptosis. Biochem. J., 319: 683-690.

Nath R et al. 1996b. Effects of calpain and ICE-like protease inhibitors on neuronal apoptosis. NeuroReport, 8: 249-256.

Neystat M, Rzhetskaya M, Oo TF, Kholodilov N, Yarygina O, Wilson A, El-Khodor BF, & Burke RE. 2001. Expression of cyclin-dependent kinase 5 and its activator p35 in models of induced apoptotic death in neurons of substantia nigra in vivo. J. Neurochem., 77(6): 1611-25.

Nguyen MD, Lariviere RC, & Julien JP. 2001. Deregulation of Cdk5 in a mouse model of ALS: toxicity alleviated by perikaryal neurofilament inclusions. Neuron, 30(1): 135-147.

Nicholson DW, Thornberry NA. 1997. Caspases: killer proteases. Trends Biochem. Sci., 22(8): 299-306.

Niethammer M, et al. 2000. NUDEL is a novel Cdk5 substrate that associates with LIS1 and cytoplasmic dynein. Neuron, 28: 697-711.

Nikolic M, Chou MM, Lu W, Mayer BJ, & Tsai LH. 1998. The p35/Cdk5 is a neuron-specific Rac effector that inhibits Pak1 activity. Nature, 395: 194-198.

Nikolic M, Dudek H, Kwon YT, Ramos YFM, & Tsai L-H. 1996. The cdk5/p35 kinase is essential for neurite outgrowth during neuronal differentiation. Genes Dev., 10: 816-825.

Nishino H, Knoll A, Buettner VL, Frisk CS, Maruta Y, Haavik J, Sommer SS. 1995. p53 wild-type and p53 nullzygous Big Blue transgenic mice have similar frequencies and patterns of observed mutation in liver, spleen and brain. Oncogene, 11: 263-70.

Nixon RA, & Sihag RK. 1991. Neurofilament phosphorylation: a new look at regulation and function. Trends Neurosci., 14(11): 501-6.

Normura M, Suzuki M, Suzuki Y, Ikeda H, Tamura J, Koike M, Jie T and Itoh G. 1996. Cyclophosphamide-induced apoptosis induces phocomelia in the mouse. Arch. Toxicol. 70: 672-677.

Nunez G, Benedict MA, Hu Y, Inohara N. 1998. Caspases: the proteases of the apoptotic pathway. Oncogene, 17(25): 3237-45.

Nylandsted J, Rohde M, Brand K, Bastholm L, Elling F, Jaattela M. 2000. Selective depletion of heat shock protein 70 (Hsp70) activates a tumor-specific death program that is independent of caspases and bypasses Bcl-2. Proc. Natl. Acad. Sci. U S A, 97(14): 7871-6.

Obst B, Schutz S, Ledig S, Wagner S, Beil W. 2002. Helicobacter pylori -induced apoptosis in gastric epithelial cells is blocked by protein kinase C activation. Microb. Pathog., 33(4): 167-75.

Oda K, Arakawa H, Tanaka T, Matsuda K, Tanikawa C, Mori T, Nishimori H, Tamai K, Tokino T, Nakamurs Y, Taya Y. 2000. p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. Cell, 102: 849-62.

Oda E, Ohki R, Murasawa H, Nemoto J, Shibue T, Yamashita T, Tokino T, Taniguchi T, Tanaka N. 2000. Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptotisis. Science, 288: 1053-8.

Ohshima T, Gilmore EC, Longenecker G, Jacobowitz DM, Brady RO, Herrup K, Kulkarni AB. 1999. Migration defects of cdk5(-/-) neurons in the developing cerebellum is cell autonomous. J Neurosci., 19(14): 6017-26.

Ohshima T, Ward JM, Huh CG, Longenecker G, Veerann A, Pant HC, Brady RO, Martin LI and Kulkarni AB. 1996. Targeted disruption of the cyclin-dependent kinase 5 gene results in abnormal corticogenesis, neuronal pathology and perinatal death. Proc. Natl. Acad. Sci. USA 93: 11173-8.

Osborne BA. 1998. Apoptotic signaling in lymphocytes. In: Lockshin RA, Zakeri Z, Tilly JL (Eds.), When Cells Die: A Comprehensive Evaluation of Apoptosis and Programmed Cell Death. Wiley-Liss, New York, pp. 245-66.

Owen-Schaub LB, Zhang W, Cusack JC, Angelo LS, Santee SM, Fujiwara T, Roth JA, Deisseroth AB, Zhang WW, Kruzel E, Radinsky R. 1995. Wild-type human p53 and a temperature-sensitive mutant induce Fas/APO-1 expression. Mol. Cell Biol., 15: 3032-40.

Paglini G, et al. 1998. Evidence for the participation of the neuron-specific Cdk5 activator p35 during laminin-enhanced axonal growth. J. Neurosci., 18: 9858-9869.

Patrick GN, Zhou P, Kwon YT, Howley PM, & Tsai LH. 1998. p35, the neuronal-specific activator of cyclin-dependent kinase 5 (Cdk5) is degraded by the ubiquitin-porteasome pathway. J. Biol. Chem., 273: 24057-24064.

Patrick GN, Zukerberg L, Nikolic M, Delamonts S, Dikkes P and Tsai LH. 1999. Conversion of p35 to p25 deregulates Cdk5 activity and promotes neurodegeneration. *Nature*, 395: 194-198.

Patzke H, & Tsai LH. 2002. Calpain-mediated cleavage of the cyclin-dependent kinase 5 activator p39 to p29. J. Biol. Chem., 277(10): 8054-8060.

Peiffer RL, McCullen R, Alles AJ and Sulik KK (1991) Relationship of cell death to cyclophosphamide-induced ocular malformations. Teratog. Carcinog. Mutagen. 11: 203-212

Pennacchio, L.A. *et al.* 1998. Progressive ataxia, myoclonic epilepsy and cerebellar apoptosis in cystatin B-deficient mice. Nat. Genet., 20: 251–8.

Pepper C, Ali K, Thomas A, Hoy T, Fegan C, Chowdary P, Kell J, Bentley P. 2002. Retinoid-induced apoptosis in B-cell chronic lymphocytic leukaemia cells is mediated through caspase-3 activation and is independent of p53, the retinoic acid receptor, and differentiation. Eur. J. Haematol., 69(4): 227-35.

Pettmann B, & Henderson CE. 1998. Neuronal cell death. Neuron, 20: 633-647.

Philpott A, Porro EB, Kirschner MW, & Tsai LH. 1997. The role of cyclin-dependent kinase 5 and a novel regulatory subunit in regulating muscle differentiation and patterning. Genes Dev., 11: 1409-1421.

Pinkoski MJ, Waterhouse NJ, Heibein JA, Wolf BB, Duwana T, Goldstein JC, Newmeyer DD, Bleackley RC, & Green DR. 2001. Granzyme B-mediated apoptosis proceeds predominantly through a bcl-2-inhibitable mitochondrial pathway. J. Biol. Chem., 276: 12060-7.

Pinon LG, Middleton G, Davies AM. 1997. Bcl-2 is required for cranial sensory neuron survival at defined stages of embryonic development. Development, 124(20): 4173-8.

Platt N, Silva RP da, & Gordon S. 1998. Recognizing death: the phagocytosis of apoptotic cells. Trends Cell Biol., 8: 365-372.

Pollack M, Phaneuf S, Dirks A, Leeuwenburgh C. 2002. The role of apoptosis in the normal aging brain, skeletal muscle, and heart. Ann. NY Acad. Sci., 959: 93-107.

Poon RY, Lew J, & Hunter T. 1997. Identification of functional domains in the neuronal Cdk5 activator protein. J. Biol. Chem., 272: 5703-5708.

Portela-Cailliau C, Hedreen JC, Price DL, & Koliatsos VE. 1995. Evidence for apoptotic cell death in Huntington disease and exitotoxic animal models. J. Neurosci., 15: 3775-3787.

Posmantur R, Kampfl A, Siman R, Liu J, Zhao X, Clifton GL, and Hayes RL. 1997. A calpain inhibitor attenuates cortical cytoskeletal protein loss after experimental traumatic brain injury in the rat. Neurosci., 77: 875-888.

Poulaki V, Mitsiades CS, Joussen AM, Lappas A, Kirchhof B, & Mitsiades N. 2002. Constitutive nuclear factor-kappaB activity is crucial for human retinoblastoma cell viability. Am. J. Pathol., 161(6): 2229-2240.

Qi L, & Sit KH. 1998. Euchromatin megabase clealvages and conjoint apoptotic-autophagic death expression with nucleolar ball-and-socket joint dislocation in human Chang liver cells arrested in S-phase by etoposide. Eur. J. Cell Biol., 77: 239-46.

Qi Z, Tang D, Zhu X, Fujita DJ, Wang JH. 1998. Association of neurofilament proteins with neuronal Cdk5 activator. J Biol Chem., 273(4): 2329-35.

Rabizadeh S, et al. 1995. Mutations associated with amyotrophic lateral sclerosis covert superoxide dismutase from an antiapoptotic gene to a proapoptotic gene: studies in yeast and neural cells. Proc. Natl. Acad. Sci. USA, 92: 3024-3028.

Ranganath RM, Nagashree NR. 2001. Role of programmed cell death in development. Int Rev Cytol., 202:159-242.

Ranson T, Vosshenrich CA, Corcuff E, Richard O, Muller W, Di Santo JP. 2003. IL-15 is an essential mediator of peripheral NK cell homeostasis. Blood, Feb. 13.

Rao RV, Castro-Obregon S, Frankowski H, Schuler M, Stoka V, del Rio G, Bredesen DE, Ellerby HM. 2002. Coupling endoplasmic reticulum stress to the cell death program. An Apaf-1-independent intrinsic pathway. J. Biol. Chem., 277(24): 21836-42.

Regula KM, & Kirshenbaum LA. 2001. p53 activates the mitochondrial death pathway and apoptosis of ventricular myocytes independent of de novo gene transcription. J. Mol. Cell. Cardiol., 33(8): 1438-1445.

Reis RA, Cabral da Silva MC, Loureiro dos Santos NE, Bampton E, Taylor JS, de Mello FG, Linden R. 2002. Sympathetic neuronal survival induced by retinal trophic factors. J. Neurobiol., 50(1): 13-23.

Reynaud K, & Driancourt MA. 2000. Oocyte attrition. Mol. Cell. Endocrinol., 163: 101-108.

Richard RA & CM Williams. 1964. Programmed cell death. II. Endocrine potentiation of the breakdown of the intersegmental muscles of silkmoths. J. Insect Physiol. 10: 643-649.

Roberts, L.R. *et al.* 1999. Cathepsins as effector proteases in hepatocyte apoptosis. Cell Biochem. Biophys., 30: 71–88.

Rokhlin OW, Glover RA, & Cohen MB. 1998. Fas-mediated apoptosis in human prostatic carcinoma cell lines occurs via activation of caspase-8 and caspase-7. Cancer Res., 58: 5870-5875.

Ronchetti A, Rovere P, Iezzi G, Galati G, Heltai S, Protti MP. 1999. Immunogenicity of apoptotic cells in vivo: role of antigen load, antigen-presenting cells, and cytokines. J. Immunol., 163: 130-136.

Rosen DR, et al. 1993. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. Nature, 362: 59-62.

Rubin E, Rottenberg H. 1982. Ethanol-induced injury and adaptation in biological membranes. Fed. Proc., 41(8): 2465-71.

Ryan KM, Ernst MK, Rice NR, Vousden KH. 2000. Role of NF-κB in p53 mediated programmed cell death. Nature, 404: 892-7.

Saatman KE, Zhang C, Bartus RT, and McIntosh TK. 2000. Behavioral efficacy of post traumatic calpain inhibition is not accompanied by reduced spectrin proteolysis, cortical lesion, or apoptosis. J. Cereb. Blood Flow Metab., 20: 66-73.

Saito T, Onuki R, Fujita Y, Kusakawa G, Ishiguro K, Bibb JA, Kishimoto T, Hisanaga S. 2003. Developmental regulation of the proteolysis of the p35 cyclin-dependent kinase 5 activator by phosphorylation. J. Neurosci., 23(4): 1189-97.

Sandal T, Stapnes C, Kleivdal H, Hedin L, and Doskeland SO. 2002. A novel extraneuronal role for cyclin-dependent protein kinase 5 (Cdk5). J. Biol. Chem., 277(23): 20783-20793.

Saraste A, & Pulkki K. 2000. Morphological and biochemical hallmarks of apoptosis. Cardiovasc. Res., 45: 528-537.

Sarin A, et al. 1995. A protease-dependent TCR-induced death inhibitors on neuronal apoptosis. NeuroReport, 8: 249-256.

Saunders Jr JW, Fallon JF. 1996. Cell death in morphogenesis. In: Locke M (Ed.), Major Problems in Developmental Biology (25th Symposium of the Society for Developmental Biology). Academic Press, New York, pp. 289.

Savill J. 1997. Recognition and phagocytosis of cells undergoing apoptosis. Br. Med. Bull., 53: 491-508.

Savill J. 1998. Apoptosis: phagocytic docking without shocking. Nature, 392: 442-443.

Savill J, Fadok V. 2000. Corpse clearance defines the meaning of cell death. Nature, 407(6805): 784-8.

Sax JK, Fei P, Murphy ME, Bernhard E, Korsmeyer SJ, & El-Deiry WS. 2002. BID regulation by p53 contributes to chemosensitivity. Nat. Cell Biol., 4(11): 842-849.

Schlegel RA, Callahan MK, Williamson P. 2000. The central role of phosphotidylserine iin the phagocytosis of apoptotic thymocytes. In: Zakeri Z, Lockshin RA, Martinez-AC. (Eds.), Mechanisms of cell death II. New York Academy of Sciences, New York, pp. 217-225.

Smith KB, & Tata JR. 1976. Cell death. Are new proteins synthesized during hormone-induced tadpole tail regression? Exp. Cell Res., 100(1): 129-146.

Schuler M, Bossy-Wetzel E, Goldstein JC, Fitzgerald P, & Green DR. 2000. p53 induces apoptosis by caspase activation through mitochondrial cytochrome c release. J. Biol. Chem., 275(10): 7337-7342.

Schweichel JU, & Merker HJ. 1973. The morphology of various types of cell death in prenatal tissues. Teratology, 7: 253-266.

Schweichel JU and Noack W (1971) Morphology and significance of physiological cell necrosis during the development of the forelimb in rat fetus. Verh. Anat. Ges. 66: 287-295.

Sen S, D'Incalci M. 1992. Apoptosis. Biochemical events and relevance to cancer chemotherapy. FEBS Lett., 307(1): 122-7.
Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW. 1997. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell, 88(5): 593-602.

Session DR, Fautsch MP, Avula R, Jones WR, Nehra A, & Wieben ED. 2001. Cyclin-dependent kinase 5 is expressed in both Sertoli cells and metaphase spermatocytes. Fertil. Steril., 75(4): 669-673.

Seubert P, Lee K, & Lynch G. 1989. Ischemia triggers NMDA receptor-linked cytoskeletal proteolysis in hippocampus. Brain Res., 492: 366-370.

Sharif-Askari E, Alam A, Rheaume E, Beresford, PJ, Scotto C, Sharma K, Lee D, DeWolf WE, Nuttall ME, Lieberman J, et al. 2001. Direct cleavage of the human fragmentation factor-45 by granzyme B induces caspase-activated DNase release and DNA fragmentation. EMBO J., 20: 3101-13.

Sharma P, Barchi JJ Jr, Huang X, Amin ND, Jaffe H, Pant HC. 1998. Site-specific phosphorylation of Lys-Ser-Pro repeat peptides from neurofilament H by cyclin-dependent kinase 5: structural basis for substrate recognition. Biochemistry, 37(14): 4759-66.

Sharma P, Sharma M, Amin ND, Albers RW, Pant HC. 1999. Regulation of cyclin-dependent kinase 5 catalytic activity by phosphorylation. Proc. Natl. Acad. Sci. U S A., 96(20): 11156-60.

Sharma P, Steinbach PJ, Sharma M, Amin ND, Barchi JJ Jr, Pant HC. 1999. Identification of substrate binding site of cyclin-dependent kinase 5. J. Biol. Chem., 274(14): 9600-6.

Shetty KT, Link WT, & Pant HC. 1993. Cdc2-like kinase from rat spinal cord specifically phosphorylates KSPXK motifs in neurofilament proteins: isolation and characterization. Proc Natl Acad Sci U S A, 90(14): 6844-8.

Shetty V, Hussaini S, Alvi S, Joshi L, Shaher A, Dangerfield B, Nascimben F, Mundle S, Allampallam K, Reddy P, Galili N, Raza A. 2002. Excessive apoptosis, increased phagocytosis, nuclear inclusion bodies and cylindrical confronting cisternae in bone marrow biopsies of myelodysplastic syndrome patients. Br. J. Haematol., 116(4): 817-25.

Shibata Y, Nishimura S, Okuyama A, Nakamura T. 1996. p53-independent induction of apoptosis by cyclin-dependent kinase inhibition. Cell Growth Differ., 7(7): 887-91.

Shimizu H, Ohgoh M, Momose Y, Nishizawa Y, & Ogura H. 2002. Massive cell death of cerebellar granule neurons accompanied with caspase-3-like protease activation and subsequent motor discoordination after intracerebroventricular injection of vincristine in mice. Neurosci., 115(1): 55-65.

Shirvan A, Ziv I, Agarwal-Mawal R, Machlyn T, Barzilai A and Melamed E. 1998. Expression of cell cycle-related genes during neuronal apoptosis: is there a distinct pattern? Neurochem. Res. 23: 767-777.

Shuang R, et al. 1998. Regulation of Munc18/syntaxin 1A interaction by cyclin-dependent kinase 5 in nerve endings. J. Biol. Chem., 273: 4957-4966.

Shung R, Zhang L, Fletcher A, Groblewski GE, Pevsner J and Stuenkel EL. 1998. Regulation of Munc-18/syntaxin 1A interaction by cyclin-dependent kinase 5 in nerve endings. J. Biol. Chem. 273: 4957-4966.

Siman R, and Noszek J. 1988. Excitatory amino acids activate calpain I and induce structural protein breakdown in vivo. Neuron, 1: 279-287.

Slee EA, Harte MT, Kluck RM, Wolf BB, Casiano CA, Newmeyer DD, Wang HG, Reed JC, Nicholson DW, Alnemri ES, Green DR, & Martin SJ. 1999. Ordering the cytochrome c-initiated caspase cascade: hierarchical activation of caspase-2, -3, -6, -7, -8, and –10 in a caspase-9-dependent manner. J. Cell Biol., 144: 281-292.

Smale G, Nichols NR, Brady DR, Finch CE, & Horton WE J. 1995. Evidence for apoptotic cell death in Alzheimer's disease. Exp. Neurol., 133: 225-230.

Sobue K, Agarwal-Mawal A, Li W, Miura Y and Paudel HK. 2000. Interaction of neuronal Cdc2-like protein kinase with microtubule-associated protein Tau. J. Biol. Chem. 275: 16673-16680

Solomon M, Belenghi B, Delledonne M, Menachem E, Levine A. 1999. The involvement of cysteine proteases and protease inhibitor genes in the regulation of programmed cell death in plants. Plant Cell, 11:431-4.

Songyang Z, et al. 1996. A structural basis for substrate specificities of protein Ser/Thr kinases: primary sequence preference of casein kinases I and II, NIMA, phosphorylase kinase, calmodulin-dependent kinase II, Cdk5, and ERK1. Mol. Cell. Biol., 16: 6486-6493.

Sorimachi H, Imajoh-Ohmi S, Emori Y, Kawasaki H, Ohno S, Minami Y, and Suzuki Y. 1989. Molecular cloning of a novel mammalian calcium-dependent protease distinct form both m- and μ-types. Specific expression of the mRNA in skeletal muscle. J. Biol. Chem., 264: 20106-20111.

Sorimachi H, Ishiura S, and Suzuki K. 1997. Structure and physiological function of calpains. Biochem. J., 328: 721-732.

Spanos S, Rice S, Karagiannis P, Taylor D, Becker DL, Winston RM, Hardy K. 2002. Caspase activity and expression of cell death genes during development of human preimplantation embryos. Reproduction, 124(3): 353-63.

Squier MK et al. 1994. Calpain activation in apoptosis. J. Cell Physiol., 159: 229-237.

Srinivasula SM, Hegde R, Saleh A, Datta P, Shiozaki E, Chai J, Lee RA, Robbins PD, Femandes-Alnemri T, Shi Y, And Alnemri ES. 2001. A conserved XIAP-interacting motif in caspase-9 and Smac/Diabolo regulates caspase activity and apoptosis. Natue, 410: 112-116.

Stadelinann C, Bruck W, Bancher C, Jellinger K, Lassmann H. 1998. Alzheimer's disease: DNA fragmentation indicates increased neuronal vulnerability, but not apoptosis. J. Neuropathol. Exp. Neurol., 57: 456-464.

Strasser A, Harris AW, Bath ML, & Cory S. 1990. Novel primitive lymphoid tumors induced in transgenic mice by cooperation between myc and bcl-2. Nature, 348(6299): 331-3.

Strasser A, Whittingham S, Vaux DL, Bath ML, Adams JM, Cory S, & Harris AW. 1991. Enforced Bcl-2 expression in B-lymphoid cells prolongs antibody responses and elicits autoimmune disease. Proc. Natl. Acad. Sci. USA, 88(19): 8661-8665.

Strocchi P, Pession A, Dozza B. 2003. Up-regulation of Cdk5/p35 by oxidative stress in human neuroblastoma IMR-32 cells. J. Cell Biochem., 88(4): 758-65.

Su F, Overholtzer M, Besser D, Levine AJ. 2002. WISP-1 attenuates p53-mediated apoptosis in response to DNA damage through activation of the Akt kinase. Genes Dev., 16(1): 46-57.

Sun F, Hayami S, Ogiri Y, Haruna S, Tanaka K, Yamada Y, Tokumaru S, Kojo S. 2000. Evaluation of oxidative stress based on lipid hydroperoxide, vitamin C and vitamin E during apoptosis and necrosis caused by thioacetamide in rat liver. Biochim. Biophys. Acta., 1500(2): 181-5.

Sun X, Lee J, Navas T, Baldwin DT, Stewart TA, & Dixit VM. 1999. RIP3, a novel apoptosis-inducing kinase. J Biol Chem., 274(24): 16871-5.

Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, Mangion J, Jacotot E, Costantini P, Loeffler M, Larochette N, Goodlett DR, Aebersold R, Siderovski DP, Penninger JM, Kroemer G. 1999. Molecular characterization of mitochondrial apoptosis-inducing factor. Nature, 397(6718): 441-6.

Sutton VR, Davis JE, Cancilla M, Johnstone RW, Ruefli AA, Sedelies K, Browne KA, & Trapani JA. 2000. Initiation of apoptosis by granzyme B requires direct cleavage of bid, but not direct granzyme B-mediated caspase activation. J. Exp. Med., 192: 1403-14.

Suzuki Y, Imai Y, Nakayama H, Takahashi K, Takino K, & Takahashi R. 2001. A serine protease, HtrA2, is released from mitochondria and interacts with XIAP, inducing cell death. Mol. Cell, 8: 613-621.

Tabuchi A, Funaji K, Nakatsubo J, Fukuchi M, Tsuchiya T, Tsuda M. 2003. Inactivation of aconitase during the apoptosis of mouse cerebellar granule neurons induced by a deprivation of membrane depolarization. J. Neurosci. Res., 71(4): 504-15.

Takagi S, Ueda Y, Hijikata M, Shimotohno K. 2001. Overproduced p73α activates a minimal promoter through a mechanism independent of its transcriptional activity. FEBS Lett., 509: 7-52.

Takahashi K, Hamada N, Yamamoto D. 2002. Ras target protein canoe is a substrate for Cdc2 and Cdk5 kinases. Arch. Insect Biochem. Physiol., 49(2):102-7.

Takahashi S, Saito T, Hisanaga SI, Pant HC, Kulkarni AB. 2003. Tau phosphorylation by cyclin-dependent kinase 5/p39 during brain development reduces its affinity for microtubules. J. Biol. Chem., 278(12): 10506-10515.

Takashima A, Murayama M, Yasutake D, Takahashi H, Yokoyama M, & Ishiguro K. 2001. Involvement of cyclin dependent kinase 5 activator p25 on tau phosphorylation in mouse brain. Neurosci. Lett., 306: 37-40.

Talanian, R.V. *et al.* 1997. Granule-mediated killing: pathways for granzyme B-initiated apoptosis. J. Exp. Med., 186: 1323–31.

Tan S, Schubert D, Maher P. 2001. Oxytosis: a novel form of programmed cell death. Curr. Top. Med. Chem., 1(6): 497-506.

Tan S, Wood M, Maher P. 1998. Oxidative stress induces a form of programmed cell death with characteristics of both apoptosis and necrosis in neuronal cells. J. Neurochem., 71(1): 95-105.

Tanaka T, Veeranna, Ohshima T, Rajan P, Amin ND, Cho A, Sreenath T, Pant HC, Brady RO, Kulkarni AB. 2001. Neuronal cyclin-dependent kinase 5 activity is critical for survival. J. Neurosci., 15;21(2):550-8.

Tang D, Young J, Li KY, Matsushita M, Tomizawa K, Matsul H, Hatase O and Wang JH. 1995. An isoform of neuronal cyclin-dependent kinase 5 (Cdk5) activator. J. Biol. Chem. 270: 26897-26903.

Tarricone C, Dhavan R, Peng J, Areces LB, Tsai LH, & Musacchio A. 2001. Structure and regulation of the Cdk5-p25nck5a complex. Molecular Cell, 8: 657-669.

Thomas DA, Du C, Xu M, Wang X, & Ley TJ. 2000. DFF45/ICAD can be directly processed by granzyme B during the induction of apoptosis. Immunity, 12: 621-32.

Thomas DA, Scorrano L, Putcha GV, Korsmeyer SJ, & Ley TJ. 2001. Granzyme B can cause mitochondrial depolarization and cell death in the absence of Bid, Bax, and Bak. Prod. Natl. Acad. Sci. USA, 98(26): 14985-14990.

Thornberry NA, Lazebnik Y. 1998. Caspases: enemies within. Science, 281(5381): 1312-6.

Thummel CS. 2001. Steroid-triggered death by autophagy. Bioessays, 23(8):677-82.

Tokuoka H, et al. 2000. Brain-derived neurotrophic factor-induced phosphorylation of neurofilament-H subunit in primary cultures of embryo rat cortical neurons. J. Cell Sci., 113: 1059-1068.

Tomioka M, Shirotani K, Iwata N, Lee HJ, Yang F, Cole GM, Seyama Y, Saido TC. 2002. In vivo role of caspases in excitotoxic neuronal death: generation and analysis of transgenic mice expressing baculoviral caspase inhibitor, p35, in postnatal neurons. Brain Res. Mol. Brain Res., 108(1-2):18-32.

Tomizawa K, Matsui H, Matsushita M, Lew J, Tokuda M, Itano T, Konishi R, Wang JH and Hatase O. 1996. Localization and developmental changes in the neuron-specific cyclin-dependent kinase 5 activator (p35nck5a) in the rat brain. Neurosci., 74: 519-529.

Town T, Zolton J, Shaffner R, Schnell B, Crescentini R, Wu Y, Zeng J, DelleDonne A, Obregon D, Tan J, & Mullan M. 2002. p35/Cdk5 pathway mediates soluble amyloid-β peptide-induced tau phosphorylation in vitro. J. Neurosci. Res., 69: 362-372.

Toyoshima F, Moriguchi T, Nishida E. 1997. Fas induces cytoplasmic apoptotic responses and activation of the MKK7-JNK/SAPK and MKK6-p38 pathways independent of CPP32-like proteases. J. Cell Biol., 139(4): 1005-15.

Trapani JA, Smyth MJ. 2002. Functional significance of the perforin/granzyme cell death pathway. Nat Rev Immunol., 2(10): 735-47.

Troy CM, Salvesen GS. 2002. Caspases on the brain. J. Neurosci. Res., 69(2): 145-150.

Tsai LH, Delalle I, Caviness VS, Chae T and Harlow E. 1994. p35 is a neural-specific regulatory subunit of cyclin-dependent kinase 5. Nature 371: 419-423.

Tsai LH, Takahashi T, Caviness VS Jr, & Harlow E. 1993. Activity and expression pattern of cyclin-dependent kinase 5 in the embryonic mouse nervous system. Dev., 119: 1029-1040.

Tseng H-C, Zhou Y, Shen Y, & Tsai L-H. 2002. A survey of Cdk5 activator p35 and p25 levels in Alzheimer's disease brains. FEBS Lett., 523: 58-62.

Tsuji K, Mizumoto K, Sudo H, Kouyama K, Ogata E, Matsuoka M. 2002. p53-independent apoptosis is induced by the p19ARF tumor suppressor. Biochem. Biophys. Res. Commun., 295(3): 621-9.

Trapani JA, Smyth MJ. 2002. Functional significance of the perforin/granzyme cell death pathway. Nat. Rev. Immunol., 2(10): 735-47.

Troy CM, Salvesen GS. 2002. Caspases on the brain. J. Neurosci. Res., 69(2): 145-50.

Turk, V. *et al.* 2001. Lysosomal cysteine proteases: facts and opportunities. EMBO J., 20: 4629–33.

Ucker DS. 1991. Death by suicide: one way to go in mammalian cellular development? New Biol., 3(2): 103-9.

Uchida T, Ishiguro K, Ohnuma J, Takamatsu M, Yonekura S and Imahori K. 1994. Precursor of Cdk5 activator, the 23 kDa subunit of tau protein kinase II: its sequence and developmental change in brain. FEBS Lett. 355: 35-40.

Urthaler F, Wolkowicz PE, Digerness SB, Harris KD, and Walker AA. 1997. MDL-28170 a membrane-permeant calpain inhibitor attenuates stunning and PKC_{ε} proteolysis in reperfused ferret hearts. Cardiovasc. Res., 35: 60-7.

Van den Haute C, Spittaels K, Van Dorpe J, Lasrado R, Vandezande K, Laenen I, Geerts H, Van Leuven F. 2001. Coexpression of human cdk5 and its activator p35 with human protein tau in neurons in brain of triple transgenic mice. Neurobiol Dis., 8(1): 32-44.

Vander Heiden MG, Thompson CB. 1999. Bcl-2 proteins: regulators of apoptosis or of mitochondrial homeostasis? Nat. Cell Biol., 1(8): E209-16.

van Loo G, Saelens X, van Gurp M, MacFarlane M, Martin SJ, Vandenabeele P. 2002. The role of mitochondrial factors in apoptosis: a Russian roulette with more than one bullet. Cell Death Differ., 9(10): 1031-42.

Varfoloweev EE, et al. 1998. Targeted disruption of the mouse caspase 8 gene ablates cell death induction by the TNF receptors, TNFRSF6/Apo1 and DR3 and is lethal prenatally. Immunity, 9: 267-276.

Varghese, J. *et al.* 2001. The role of calpain in caspase activation during etoposide induced apoptosis in T cells. Eur. J. Immunol., 31: 2035–41.

Vartiainen N, Keksa-Goldsteine V, Goldsteins G, & Koistinaho J. 2002. Aspirin provides cyclin-dependent kinase 5-dependent protection against subsequent hypoxia/reoxygenation damage in culture. J. Neurochem., 82(2): 329-35.

Vercammen D, Beyaert R, Denecker G, Goossens V, Van Loo G, Declercq W, Grooten J, Fiers W, Vandenabeele P. 1998. Inhibition of caspases increases the sensitivity of L929 cells to necrosis mediated by tumor necrosis factor. J. Exp. Med., 187(9): 1477-85.

Verhagen AM, Vaux DL. 2002. Cell death regulation by the mammalian IAP antagonist Diablo/Smac. Apoptosis, 7(2): 163-6.

Vermeulen K, Strnad M, Havlicek L, Van Onckelen H, Lenjou M, Nijs G, Van Bockstaele DR, Berneman ZN. 2002. Plant cytokinin analogues with inhibitory activity on cyclin-dependent kinases exert their antiproliferative effect through induction of apoptosis initiated by the mitochondrial pathway: determination by a multiparametric flow cytometric analysis. Exp. Hematol., 30(10): 1107-14.

Vincent I, Jicha G, Rosado M, and Dickson DW. 1997. Aberrant expression of mitotic cdc2/cyclin B1 kinase in degenerating neurons of Alzheimer's disease brain. *J. Neurosci.*, 17: 3588-3598.

Volbracht C, Leist M, Nicotera P. 1999. ATP controls neuronal apoptosis triggered by microtubule breakdown or potassium depravation. Mol. Med., 5: 477-489.

Vorburger SA, Pataer A, Yoshida K, Barber GN, Xia W, Chiao P, Ellis LM, Hung MC, Swisher SG, & Hunt KK. 2002. Role for the double-stranded RNA activated protein kinase PKR in E2F-1-induced apoptosis. Oncogene, 21(41): 6278-6288.

Vousden KH, Lu X. 2002. Live or let die: the cell's response to p53. Nat. Rev. Cancer, 2(8): 594-604.

Vyas S, Bechade C, Riveau B, Downward J, Triller A. 2002. Involvement of survival motor neuron (SMN) protein in cell death. Hum. Mol. Genet., 11(22): 2751-64.

Wagner M, Hampel B, Bernhard D, Hala M, Zwerschke W, & Jansen-Durr P. 2001. Replicative senescence of human endothelial cells in vitro involves G1 arrest, polyploidization and senescence-associated apoptosis. Exp. Gerontol., 36(8): 1327-1347.

Wang KKW. 2000. Calpain and caspase: can you tell the difference? TINS, 23(1): 20-26.

Wang KKW, Posmantur R, Nath R, McGinnis K, Whitton M, Talanian RV, Glantz SB, and Morrow JS. 1998. Simultaneous degradation of αII- and βII-spectrin by caspase 3 (CPP32) in apoptotic cells. J. Biol. Chem., 273(35): 22490-7.

Warchol ME, Matsui JI, Simkus EL, Ogilive JM. 2001. Ongoing cell death and immune influences on regeneration in the vestibular sensory organs. Ann. N. Y. Acad. Sci., 942: 34-45.

Waterhouse, N.J. *et al.* 1998. Calpain activation is upstream of caspases in radiation-induced apoptosis. Cell Death Differ., 5: 1051–61.

Weil M, Jacobson MD. Coles HSR, Davies TJ, Cardner RL, Raff KD, & Raff MC. 1996. Constitutive expression of the machinery for programmed cell death. J. Cell Biol., 133: 1053-1059.

Westwood G, Dibling BC, Cuthbert-Heavens D, Burchill SA. 2002. Basic fibroblast growth factor (bFGF)-induced cell death is mediated through a caspase-dependent and p53-independent receptor pathway. Oncogene, 21(5): 809-24.

Whitaker SJ. 1992. DNA damaged by drugs and radiation: what is important and how is it measured? Eur. J. Cancer, 28: 273-6.

Worchol ME, Matsui JI, Simlus EL, Ogilive JM. 2001. Ongoing cell death and immune influences on regeneration in the vestibular sensory organs. Ann. NY Acad. Sci., 942: 34-45.

Wright, S.C. *et al.* 1998. Chemotherapeutic drug activation of the AP24 protease in apoptosis: requirement for caspase 3-like-proteases. Biochem. Biophys. Res. Commun., 245: 797–803.

Wu GS, Ding Z. 2002. Caspase 9 is required for p53-dependent apoptosis and chemosensitivity in a human ovarian cancer cell line. Oncogene, 21(1): 1-8.

Wu X, Daniels T, Molinaro C, Lilly MB, Casiano CA. 2002. Caspase cleavage of the nuclear autoantigen LEDGF/p75 abrogates its pro-survival function: implications for autoimmunity in atopic disorders. Cell Death Differ., 9(9):915-25.

Xiang H, Kinoshita Y, Knudson CM, Korsmeyer SJ, Schwartzkroin PA, & Morrison RS. 1998. Bax involvement in p53-mediated neuronal cell death. J. Neurosci., 18: 1363-1373.

Xue L, Fletcher GC, & Tolkovsky AM. 1999. Autophagy is activated by apoptotic signaling in sympathetic neurons: an alternative mechanism of death execution. Mol. Cell Neurosci., 14: 180-198.

Yaar M, et al. 1997. Binding of beta-amyloid to the p75 neurotrophin receptor induces apoptosis. A possible mechanism for Alzheimer's disease. J. Clin. Invest., 100: 2333-2340.

Yakovlev AG, Knoblach SM, Fan L, Fox GB, Goodnight R, & Faden AI. 1997. Activation of CPP32-like caspase contributes to neuronal apoptosis and neurological dysfunction after traumatic brain injury. J. Neurosci., 17: 7415-7424.

Yan SD, et al. 1996. RAGE and amyloid-β peptide neurotoxicity in Alzheimer's disease. Nature, 382: 685-691.

Yang A, et al. 1998. p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing and dominant-negative activities. Mol. Cell, 2: 305-16.

Yang A, et al. 1999. p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. Nature, 398: 714-8.

Yang A, Kaghad M, Caput D, McKeon F. 2002. On the shoulders of giants: p63, p73 and the rise of p53. Trends Genet., 18(2): 90-5.

Yang S, Delagdo R, King SR, Woffendin C, Barker CS, Yang Z-Y, Xu L, Nolan GP, Nabel GJ. 1999. Generation of retroviral vector for clinical studies using transient transfection. Human Gene Therapy, 10: 123-32.

Yeh WC, Pompa JL, et al. 1998. Essential for embryo development and signaling from some, but not all, inducers of apoptosis. Science, 279: 1954-8.

Yin Y, Terauchi Y, Solomon GG, Aizawa S, Rangarajan PN, Yazaki Y, Kadowaki T, Barrett JC. 1998. Involvement of p85 in p53-dependent apoptotic response to oxidative stress. Nature, 391(6668): 707-10.

Yokota M, Tani E, Tsubuki S, Yamaura I, Nakagaki I, Hori S, and Saido TC. 1999. Calpain inhibitor entrapped in liposome rescues ischemic neuronal damage. Brain Res., 819: 8-14.

Yonish-Rouach E, Resnitzky D, Lotem J, Sachs L, Kimchi A, & Oren M. 1991. Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. Nature, 352: 345-347.

Yuan J, Shaham S, Ledoux S, Ellis HM, & Horvitz HR. 1993. The C. elegans cell death gene Ced-3 encodes a protein similar to mammalian interleukin-1beta-converting enzyme. Cell, 75: 641-652.

Yuen P-W and Wang KKW. 1998. Calpain inhibitors, novel neuroprotectants and potential anticataractiv agents. Drugs Future, 23: 741-749.

Yoshida K, Sorimachi Y, Fujiwara M, and Hironaka K. 1995. Calpain is implicated in rat myocardial injury after ischemia or reperfusion. Jpn. Circ. J., 59: 40-48.

Zakeri Z, & Ahuja HS. 1994. Apoptotic cell death in the limb and its relationship to pattern formation. Biochem. Cell Biol., 72: 603-613.

Zakeri Z, Lockshin RA. 2002. Cell death during development. J. Immun. Methods, 265: 3-20.

Zakeri Z, Quaglino D, & Ahuja HS. 1994. Apoptotic cell death in mouse limb and its suppression in the hammertoe mutant. Dev. Biol., 165: 294-297.

Zakeri ZF, Quaglino D, Latham T, Lockshin RA. 1993. Delayed internucleosomal DNA fragmentation in programmed cell death. FASEB J., 7(5): 470-8.

Zhang C, Siman R, Xu YA, Mills AM, Frederick JR, Neumar RW. 2002. Comparison of calpain and caspase activities in the adult rat brain after transient forebrain ischemia. Neurobiol. Dis., 10(3): 289-05.

Zhang Q, Ahuja HS, Zakeri Z and Wolgemuth DJ. 1997. Cyclin-dependent kinase 5 is associated with apoptotic cell death during developmental and tissue remodeling. Dev. Biol. 183: 222-233.

Zhang J, and Johnson GVW. 2000. Tau protein hyperphosphorylation in a site-specific manner in apoptotic neuronal PC12 cells. *J. Neurochem.*, 75: 2346-2357.

Zhang J, Krishnamurthy PK, & Johnson GVW. 2002. Cdk5 phosphorylates p53 and regulates its activity. J. Neurochem., 81: 307-313.

Zheng TS, Hunot S, Kuida K, & Flavell RA. 1999. Caspase knockouts: matters of life and death. Cell Death Diff., 6: 1043-1053.

Zhu Y. 2002. The study of the role of cyclin-dependent kinase 5 (Cdk5) during cell death. A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy to the City University of New York.

Zhu Y, Lin L, Kim S, Quaglino D, Lockshin RA, and Zakeri Z. 2002. Cyclin dependent kinase 5 and its interacting proteins in cell death induced in vivo by cyclophosphamide in developing mouse embryos. *Cell Death Diff.*, 9(4): 421-430.

Zhu DM and Uckun FM. 2000. Cathepsin inhibition induces apoptotic death in human leukemia and lymphoma cells. Leuk. Lymphoma, 39: 343–54.

Zou H, Li Y, Liu X, Wang X. 1999. An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. J. Biol. Chem., 274(17): 11549-56.

Zukerberg LR, et al. 2000. Cables links Cdk5 and c-Abl and facilitates Cdk5 tyrosine phosphorylation, kinase upregulation, and neurite outgrowth. Neuron, 26: 633-646.