# ACTIVITY AND REGULATION OF CYCLIN-DEPENDENT KINASE 5 (CDK5) DURING CELL DEATH

by

YIXIA YE

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| _____ | _____ |
| Date | Chair of Examining Committee<br>Dr. Zahra Zakeri, Queens College of CUNY |
| _____ | _____ |
| Date | Executive Officer<br>Dr. Edward Kennelly, Biochemistry Doctoral Program |
| | _____<br>Dr. Raymond Birge, University of Medicine & Dentistry of NJ |
| | _____<br>Dr. Henry Tiedge, Downstate Medical Center of SUNY |
| | _____<br>Dr. Cathy Savage-Dunn, Queens College of CUNY |
| | _____<br>Dr. Wilma Saffran, Queens college of CUNY |

Supervisory Committee

THE CITY UNIVERSITY OF NEW YORK

Abstract

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Advisor: Dr. Zahra Zakeri

Cell death is an important element of development, tissue maintenance and disease. This biological process is tightly controlled by different cell death signaling pathways and a number of genes have been implicated in the regulation of cell death. Cyclin-dependent kinase 5 (Cdk5), identified due to its sequence homology to Cdc2, is a gene that is activated during cell death. The aim of this study is to investigate the roles of Cdk5 by examining the relationship of Cdk5 to different pathways involved in cell death, then evaluate the regulation of Cdk5 activation during cell death and whether Cdk5 activity is required for the induction of cell death.

By using different models in which massive cell death was induced by different toxins, including cyclophosphamide (CP)-treated mouse embryos, cyclohexamide (CHX) or camptothecine (CPT)-treated mouse embryonic fibroblast cell lines and measuring the modulation of different cell death related genes, such as caspase-3, calpain and lysosomal proteases, cathepsins, we have shown that the activation of Cdk5 is dispensible of the activity of caspase-3, calpain, and the lysosomal proteases, cathepsins. Furthermore, the finding that Cdk5 is activated without the generation of p25, an activator of Cdk5 during cell death, suggests that other activators for Cdk5 activation exist during cell death. Additionally, Inhibition of the production of Cdk5 activators, p25 and p29, by calpain

inhibitor or down-regulation of Cdk5 activity by RNAi reveals Cdk5-independent cell death; and the mode of cell death is not altered in these situations. We therefore conclude that Cdk5 activation during cell death is a result of cell death rather than an initiator of cell death.

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## Chapter I. SIGNIFICANCE OF CELL DEATH

1. Importance of cell death in development
2. Effect of teratogens on cell death in development
3. Importance of cell death in pathogenesis

Cell death is an important part of the normal life of an organism, occurring in almost all multicellular organisms throughout their life spans. Cell death was first recognized as a programmed physiological event in the nervous system of toad embryos in 1842 (Vogt, 1842). Today, cell death has become one of the most fashionable topics of research due to its importance in different aspects of living organisms from the formation of limbs during embryogenesis to the peeling of sunburned skin. Many genes have been shown to have a role in regulation of cell death; among them are the cell cycle genes and cell cycle dependent kinases. Cyclin dependent kinase 5 is one of these genes, which this laboratory has shown to be activated during cell death in many situations. However we do not know how Cdk5 is regulated and how its regulation affects cell death. The experiments in this thesis address these points and in this regard our introduction will present information on the importance and types of cell death as well as the different pathways that are involved in regulating cell death. We will also discuss how cell cycle genes and specifically Cdk5 are involved.

## 1. Importance of cell death in development

Like cell proliferation and differentiation, cell death, especially programmed cell death (PCD), which is the type of cell death that results from a sequence of predictable events and activation of specific genes, also plays important roles during development (Lockshin & Zakeri, 2004). Cell death has a role in the regulation of cell number. The balance between cell death and cell proliferation is very important for the homeostasis of different tissues not only during development but also during adulthood. It is estimated that around 10 billion cells are made every day to balance apoptosis in the adult human

body to maintain homeostasis (Renehan et al., 2001; Elmore, 2007). During development, cell death and mitoses can increase significantly. Cell death is necessary for removing unwanted cells and critical for controlling cell numbers, patterning of tissue, and shaping organs (Coucouvanis et al., 1995; Chanoine & Hardy, 2003).

Cell death starts as early as the 16-cell and blastula stage of mammalian embryos and it appears in all of the stages of development (Hardy et al., 1989; Penaloza et al., 2006). Cell death plays an important role in removal of the presumptively excess cells in different organs during development, and in sculpting structures and eliminating structures during embryogenesis, metamorphosis, and puberty across species. In a common scheme of development, excess cells are produced, and many of the original cells die through PCD. In the nervous system, about half of neurons produced during neurogenesis die before central nervous system (CNS) maturation (Lossi & Gambino 2008). For example, only about fifty percent of the motor neurons survive and function prior to birth during chick embryogenesis (Buss & Oppenheim, 2004). In GAD67/GFP knock-in mice, the stellate and basket cells that proliferate in white matter undergo apoptosis during their migration from white matter to the molecular layer (Yamanaka et al., 2004). In the reproductive system, the final number of oocytes available for reproduction of the next generation is defined at birth in mammals; eighty percent of the original germ cells in human females are lost by apoptosis by the time of birth (Reynaud & Driancourt, 2000; Pepling et al., 2006). Studies from the effect of low-dose radiation in somatic cells have showed that apoptosis of male germ cells is required for normal spermatogenesis in spermatocytes of mouse and also functions in removal of abnormal or superfluous cells at specific checkpoints (Liu et al., 2007). During sex differentiation of

mammals, the Mullerian duct in males and Wolffian duct in females degenerate by apoptosis (Lee et al., 1998). In the immune system, more than ninety-five percent of T-lymphocytes undergo apoptosis during maturation (Song et al., 1996) and immature B-lymphocytes that cannot produce IgM die through apoptosis (Compton 1992). Cell death also plays an essential role in the education of immune cells in the thymus and the bone marrow, where auto-reactive cells are eliminated (Feig & Peter, 2007).

Cell death is also involved in the changes of body plan of different species. In tadpoles, tightly controlled cell death results in the degeneration of the tail and the disappearance of the gill (Jeffery et al., 2002). The elimination of the surplus cells by PCD is necessary for the formation of the digits of the limbs of mouse and bird by the removal of the interdigital webs (Zakeri & Ahuja, 1994; Ameisen, 1996; Weatherbee et al., 2006). In cardiac morphogenesis, cell death is essential in generation of the four-chambered architecture of the heart (Abdelwahid et al., 2002). Cell death also hollows out solid structures to create cavities. For example, the formation of the preamniotic cavity in early mouse embryos is completed by the death of the ectodermal cells in the core of the developing embryos (Jacobson et al., 1997; Bodle et al., 2007). The size of organs and organisms is maintained by the regulation of the balance between cell proliferation and cell death, so that cell death is essential for size control and renewal of cell population. As is the case in animals, PCD is an essential process during growth and development in plants; it is involved in the xylogenesis, aerenchyma formation, petal senescence, and endosperm development. PCD also occurs in the leaves of several species to form holes or to allow fronds to separate (Hoeberichts & Woltering, 2002). Recent studies in *Drosophila* have shown that Hippo signaling pathway is implicated in

the regulation of organ size during development by coordinating regulation cell death and cell proliferation (Dong et al., 2007).

Developmental cell death is a process tightly controlled by death machinery composed of different genes that will be discussed in detail later. In this thesis, we will use developing mouse embryos to explore the regulation of cell death related genes, such as caspase-3 and Cdk5, during cell death in development.

## 2. Effect of teratogens on cell death in development

Cell death is detected in many organs during normal development as mentioned above; however excessive cell death or inappropriate cell death also contributes to abnormal embryonic development. Chemical and physical agents that disrupt development result in structural malformations and deficits in experimental animals (Shepard, 2001).

Teratogens are agents that interfere with the normal embryonic development and result in the malformation of embryos or birth defects, often associated with inappropriate cell death (Mirkes & Little 1998). Cell death induced by teratogens often occurs in the areas where normal programmed cell death usually occurs (Menkes et al., 1977). Teratogens, such as ionizing radiation, periods of hypoxia, infections and chemicals (Brill et al., 1999) can operate by different mechanisms. For example, hypoxia is associated with cell death in specific regions and subsequent malformation, such as cleft lip formation; and the relationship of hypoxia-induced cell death to energy requirement is being explored (Webster et al., 2006). Rubella infection during pregnancy can cause fetal damage, including congenital heart disease, neurological damage and

hearing defect, due to the damage to the wall of blood vessel and lining of the heart and organ infarction and ischemia subsequently (Webster 1998; Minussi et al., 2008). Of the chemical teratogens, retinoic acid (RA) and cyclophosphamide (CP), the two major teratogens used for the studies of cell death, will be discussed below.

Retinoic acid (RA), the metabolite of vitamin A and its derivatives, is known to modulate cell proliferation, differentiation and death through binding to nuclear receptors (Sporn et al., 1994). Retinoic acid receptors, which bind retinoid and regulate transcription (Mangelsdorf et al., 1994), are involved in the 20-epi-vitamin-D3 induced apoptosis and inhibition of proliferation (Gumireddy et al., 2003). Retinoid is also a potent teratogen that can induce congenital defects in all vertebrate species at teratogenic dosage. Exogenous retinoic acid can increase apoptotic cell death in the interdigital regions of mouse limb (Zakeri et al., 1993; 1994). Apoptosis induced by all-trans retinoic acid in the early developing eye of vertebrates is associated with the expression of caspase-3 and HSP110 (Gashequ et al., 2007). In rat models, retinoic acid is also associated with the abnormal development of spinal cord in spina bifida through downregulation of microRNAs (Zhao et al., 2008). Very recently, it has been found that poly (ADP-ribose) polymerase (PARP)/ c-Jun N-terminal kinase 1 (JNK1) cascade is involved in the cell death induced by TNFα and amplified by all-trans retinoic acid (ATRA) in APL cells (Mathieu et al., 2008).

Cyclophosphamide (CP) is a cytotoxic agent widely used in treatment of malignancies (Mihelic et al., 2007), progressive autoimmune diseases (Schieppati & Remuzzi 2008) and transplant rejection (Almuti et al., 2007) and it is also frequently used as an experimental teratogen in animals. Embryos treated with CP displayed apoptosis in

target organs, such as limbs, head and regions of craniofacial anomalies (Torchinsky et al., 1995; Wang et al., 2008). Since CP can induce massive cell death in developing mouse embryos, CP-treated embryos represent a good model for studying the mechanism of cell death and the roles of cell death genes during cell death. Native cyclophosphamide is inactive and is metabolized to cytotoxic substances, such as phosphoramide mustard and acrolein, by cytochrome P450 mono-oxygenase in the liver (Moore 1991; Mingoia et al., 2007). The cytotoxicity of CP is thought to result from the production of mutations, DNA-strand breaks, chromatin aberration, micronuclei, and sister chromatid exchanges (Selvakumar et al., 2006). During organogenesis, exposure to CP induces a variety of malformations, including open eyes, cleft palate, phocomelia, adactyly, oligodactyly, syndactyly, polydactyly, kinky tail, exencephaly or hydrocephaly, as well as disturbance in skeletal ossification (Gibson & Becker, 1968; Hales 1981). Neurocytotoxic effects of cyclophosphamide after neural tube closure result from cyclophosphamide-induced neuronal apoptosis and DNA damage (Xiao et al., 2007). The activated analog of CP, 4-hydroperoxycyclophosphamide, can cause growth delay and malformation in the cultured limb, and increased apoptosis in the interdigital spaces and apical endodermal ridge, where apoptosis occurs normally (Huang & Hales 2002). CP can induce massive cell death in treated embryos, and the cell death induced by CP is apoptotic by the criteria of DNA fragmentation, activation of caspase-3, cleavage of poly(ADP-ribose) polymerase (PARP) and activation of caspase-9 in early postimplantation mouse embryos (Schwartz & Waxman, 2001; Little & Mirkes, 2002). CP is involved in apoptotic cell death by activating the cell death regulating genes, including caspase-3, 9, 7, and 6, cathepsin D,

and it can also induce the release of cytochrome c from the mitochondria (Little et al., 2003; Ohtani et al., 2006; Pekar et al., 2007).

Previous data from our lab have shown that CP induces massive apoptotic cell death in mouse embryos and that cyclin-dependent kinase 5 (Cdk5) is expressed during apoptotic cells. The kinase activity of Cdk5 is markedly elevated during CP-induced cell death. Since CP is involved in the apoptotic cell death signaling pathways related caspase-3 activation and Cdk5 activity is also increased in CP-induced cell death, the model of CP-treated embryos is useful to explore the activity of Cdk5 with caspase-3 activation in neuronal cell death induced by CP.

### 3. Importance of cell death in pathogenesis

Cell death is a tightly regulated process and deregulation of cell death can lead to pathologies, including neurodegenerative diseases such as Alzheimer's disease (AD) (Campbell & Gowran 2007), Parkinson's disease (PD) (Olanow 2007), Huntington's disease (HD) (Zuchner & Brundin 2007), Amyotrophic Lateral Sclerosis (ALS) (Beal 2007), autoimmune diseases, cancers, infections, and infarction (Maniati et al., 2007).

Excessive cell death is thought to play an important role in neurodegenerative diseases, neuronal loss, and synapse loss by cell death in neurodegenerative diseases (Bredesen et al., 2006). In Alzheimer's disease, apoptosis in neurons, glia and microglia is induced by amyloid β, which is processed from amyloid precursor protein and deposited extracellularly as plaques, by causing oxidative stress, triggering Fas ligand expression and activating TNF-R1 (Ethell & Buhler, 2003; Culpan et al., 2007). Parkinson's disease (PD) is characterized by the loss of dopamine neurons by cell death

in substantia nigra pars comparta and is related to oxidative stress and mitochondrial dysfunction (Lee et al., 2008). Inhibition of cell death can inhibit neurodegeneration. For example, expression of anti-apoptotic protein delayed symptom onset and increased lifespan in a transgenic model of ALS and pro-apoptotic gene inhibition *in vivo* delayed the degeneration of neurons in both ALS and HD transgenic mouse models (Bredesen et al., 2006).

Also, lower than normal cell death is pathogenic. Cancer can be an example of either overproliferation of cells or decreased removal of cells. In tumor cells, the pro-apoptotic proteins are often down-regulated or mutated and anti-apoptotic proteins are expressed, resulting in decrease of cell death. The expression of pro-apoptotic protein or anti-apoptotic protein is regulated by the tumor suppressor gene, p53 (Miyashita et al., 1994; Elmore 2007). Decreased cell death can also lead to autoimmune diseases. Autoimmunity is considered as a multi-step process, with programmed cell death being a key mechanism to regulate immune system function. Defects in apoptotic death pathways may contribute to the development of autoimmune response in susceptible individuals (Rashedi et al., 2007). Autoimmune lymphoproliferative syndrome (ALPS) is caused by insufficient apoptosis of T cells and overproliferation of B cells resulting in multiple autoimmune syndromes and excess immunoglobin production (Worth et al., 2006).

The involvement of cell death in pathology further argues for the importance of tightly controlled cell death in a healthy life. In order to gain control of cell death, many signaling pathways need to be regulated and coordinated. Some of these pathways will be discussed in more detail in the following chapter.

## Chapter II. Mechanisms of Cell Death

1. Apoptotic cell death
2. Autophagic cell death
3. Necrotic cell death
4. Other types of cell death

Cell death can occur through different pathways leading to different morphological and chemical changes. Based on the morphological and biochemical criteria, cell death has been classified into three major forms: apoptosis, autophagy, and necrosis.

### 1. Apoptotic cell death

The term "Programmed Cell Death" (PCD) was first used to describe cell death in a predictable place and time during development by Lockshin and Williams in 1964 (Lockshin &Williams, 1964). Subsequently, Kerr and his colleagues coined "apoptosis" to depict the physiological cell death with particular morphology in 1972, such as membrane blebbing, chromatin fragmentation (subsequently ascribed to internucleosomal DNA cleavage), nuclear condensation, cell shrinkage, and membrane-bound apoptotic body formation (Kerr et al., 1972; Saraste & Pulkki, 2000). During apoptosis, the cells round up and condense with loss of volume but maintain the integrity of the cell membrane. Nuclear margination occurs, in which chromatin coalesces into masses along the nuclear membrane. Meanwhile, the cytoplasm fragments and the plasma membrane blebs, resulting in the formation of apoptotic bodies. The apoptotic dying cells separate from their neighbors and the elimination of the apoptotic bodies is accomplished through phagocytosis of the bodies by adjacent cells and/or macrophages (Malhi et al., 2006). Apoptosis is divided into three distinct phases: initiation phase, effector phase and degradation phase (Hail et al., 2006). The initiation phase is a triggering event dependent on the cell type and the stimuli. Many specific stimuli, such as death receptor ligands including TNF-α (tumor necrosis factor-α) and Fas ligand; and DNA damage including

ionizing radiation and chemotherapeutic agents as well as growth factor withdrawal can initiate apoptotic cell death (Bredesen, 2000; Green & Kroemer, 2004). In the effector phase, biochemical events activate proteases and nucleases. The stimuli listed above most often result in the activation of a cascade of caspases, cysteine-aspartate proteases, which are a hallmark of apoptosis and are responsible for the degradation of critical cellular proteins as discussed below. In the degradation phase, caspases, cathespins and calpains cleave the proteins and eventually DNA, leading to apoptotic cell death. These proteolytic enzymes are involved in the cell death signaling apoptotic pathways and the interaction of these genes as well as the level of the expression and activation of the proteins regulate apoptotic pathways. Two distinct evolutionarily conserved pathways have been proposed to mediate apoptosis: the extrinsic pathway, which is activated by the binding of death ligands to their receptors at the plasma membrane and the intrinsic pathway, which is initiated by a number of insults, such as DNA damage, loss of growth factors, hypoxia and oxidative stress (Gustafsson & Gottlieb, 2007). The signaling pathways and the genes involved in these cell death signaling pathways will be discussed in detail below.

**Caspases** Caspases are a family of cysteine aspartate-specific proteases, which have central functions in apoptotic and inflammatory signaling pathways. The activation of execution caspases is considered as the apoptotic commitment point in the signaling pathways. The role of caspases during cell death has been extensively investigated and the importance of caspases in cell death has been clearly established.

In the early 1990s, genetic and biochemical studies of apoptotic cell death in *C. elegans* demonstrated that specific proteases were involved in apoptotic execution and that these proteases were homologous to human interleukin-1 β converting enzyme (caspase-1, or ICE) (Yuan et al., 1993; Cerretti et al., 1992). Subsequent studies identified 13 mammalian proteases that were related to the proteases in *C. elegans*, and they were termed caspase proteases along with ICE (Alnemri et al., 1996; Thornberry & Lazebnik, 1998).

Caspases are an evolutionarily conserved family of cysteine proteases that cleave the target proteins at sites C-terminal to specific aspartic acid residues in apoptotic and inflammatory signaling pathways (Li & Yuan 1999; Lamkanfi et al., 2002). All caspases are synthesized as zymogens with very low enzymatic activity. Each procaspase (30-50kDa) is composed of three domains: an N-terminal prodomain and other two domains consisting of a large subunit (20kDa) and a small subunit (10kDa). The N-terminal of procaspases is proteolytically processed to produce active caspases (Degterev et al., 2003; Fuentes-Prior & Salvesen 2004; Gogvadze & Orrenius 2006). The caspases derived from precursors with long prodomain (caspases-2, -8, -9, and -10) are termed "initiator" or "upstream" caspases because they are the first line of activation after a death signal triggers apoptosis and they are able to activate downstream caspases. Caspases-3, -6, and -7 with short prodomains are named "effector" or "downstream", and their activation by the initiator caspases results in irreversible cell death by cleaving specific vital substrates, such as poly(ADP)-ribose polymerase (PARP), gesolin and lamins. A number of caspases, including caspases -1, -4, -5, -11, and -14, which also have long prodomain, are related to the activation of proinflammatory cytokines and their activation may not

necessarily involve apoptosis (Li & Yuan 1999; Kidd et al., 2000; LeBlanc, 2003; Kumar 2007). Activated caspases cleave many cellular proteins, including proteins responsible for cell cycle regulation (e.g. RB, MDM2, Los et al., 2001); DNA damage recognition and repair proteins (e.g. DNA-PK, PARP, Martin & Green 1995) and proteins regulating cellular structure (e.g. actin and lamins, Moretti et la., 2002; Croft et al., 2005). Caspase-3 and caspase-7 double knockout (DKO) mice die rapidly after birth and display defects in heart development. Mouse embryo fibroblast (MEF) cells from caspase-3 and –7 DKO mice are resistant to cell death induced by UV, staurosporine, FasL and TNF, which suggests that the function of at least one of caspase-3 and caspase-7 is required for cell death (Lakhani et al., 2006). The activation of caspases is tightly regulated in various ways. For example, there are many inhibitor of apoptosis proteins (IAPs) in many organisms. The mammalian IAPs include XIAP, cIAP-1 and -2, NAIP, ML-IAP, ILP-2 and survivin, among which XIAP, cIAP-1 and -2 can physically interact with caspases to inhibit mature caspase-3, -7, and -9 (Vaux & Silke 2005; McStay et al., 2008). FLIP is a key regulator of caspase-8 activation in the extrinsic pathway and FLIP is necessary for protecting cells from caspase-8 mediated apoptosis induced by TNFR family members (Yeh et al., 2000; Micheau & Tschopp, 2003).

Caspases are also involved in differentiation and enucleation processes, such as lens cell differentiation, erythrocyte and platelet formation and the terminal differentiation of keratinocytes. Many studies from knockout mice elucidated the importance of caspase activity in development. Caspase-3 activity is required for the maintenance of lens transparency, since cataracts at the anterior lens pole are found in caspase-3 knockout mice (Zandy et al., 2005; Lamkanfi et al., 2007). In caspase-9 -/-

mice, hyperplasia in the brain resulted from decreased apoptosis and defective neural tube closure in the hindbrain region (Hakem et al., 1998; Kuida et al., 1998; Oppenheim et al., 2001). Caspase-8 null mice display impaired heart muscle development, and caspase-8 is required for macrophage differentiation (Varfolomeev et al., 1998; Kang et al., 2004). During apoptosis, the activation of caspases is regulated by mitochondrial proteins. Mitochondrial outer membrane permeabilization (MOMP) results in the release of cytochrome c, which activates caspase-9 in cytosol, and caspase-9 then activates caspase-3. The activated caspase-8 cleaves pro-apoptotic protein Bid and tBid can mediate the release of cytochrome c from the mitochondria (Byun et al., 2008). Caspases -3, -6, and -7 also stimulate the release of cytochrome c when added to isolated mitochondria in vitro (Bossy-Wetzel & Green, 1999; Chuang et al., 2007). Thus, the activation of caspases plays an essential role in cell death and regulation of their activation initiates the execution of the doomed cells. Cdk5 is a unique member of cyclin dependent kinase family, the activity of which has been related to cell differentiation and cell death. In view of the roles that caspases play during developmental cell death, a relationship between Cdk5 and caspase-3 may exist during cell death. In this thesis, we will evaluate the dependency of Cdk5 activity on caspase-3 during neuronal cell death in developing mouse embryos.

**Bcl-2 family members** Bcl-2 (B-cell leukemia/lymphoma-2), the mammalian homologue of C. elegans CED-9, was originally described as a proto-oncogene found at the chromosomal breakpoint of t(14:18) in B-lymphomas (Bakhshi et al., 1985). In 1990, the Korsmeyer group found that overexpression of Bcl-2 blocked apoptotic death in pro-

B-lymphocyte cell line (Hockenbery et al., 1990). In 1997, the proteins in this family were recognized as important for the release of cytochrome c from the mitochondria during cell death. For example, Bcl-2 prevents the efflux of cytochrome c from mitochondria and Bax can stimulate the release of cytochrome c (Yang et al., 1997; Kluck et al., 1997; Manon et al., 1997).

The more than 30 identified proteins in the Bcl-2 family have been classified into three subfamilies according to their sequence homology in four α-helical segments called BH1, BH2, BH3, and BH4 (Tsujimoto & Shimizu, 2000; Danial et al., 2003). The members of the highly conserved anti-apoptotic subfamily comprising Bcl-2, Bcl-xL, Bcl-w, Mcl-1 A1 and also Bcl-B in humans contain all four BH domains, of which BH1, BH2 and BH3 domains can form the pocket that can bind with the BH3 domain of other family members (Youle & Strasser 2008). The anti-apoptotic proteins protect cells from death. For instance, Bcl-2 is essential for the survival of kidney, melanocyte stem cells, and mature B and T lymphoid cells (Nakayama et al., 1993; Veis et al., 1993; Sohn et al., 2007), and Mcl-1 is obligatory for the survival of the zygote (Rinkenberger et al., 2000). The other two pro-apoptotic subfamilies were identified as Bcl-2 binding proteins that promote cell death. The Bax-like pro-apoptotic subfamily members consisting of Bax, Bak, Mtd (Bok) and Bcl-Rambo have three conserved BH domains, BH1, BH2 and BH3. "BH3-only proteins" including Bik (Nbk), Bad, Bid, Bim, Hrk, Noxa, Blk, Bnip3, Bnip3L, Puma, p193, Bmf and Bcl-G represent the third family and demonstrate that BH3 is essential for the killing function of pro-apoptotic proteins (Tsujimoto, 2003; Opferman & Korsmeyer 2003; Willis & Adams, 2005; Moldoveanu et al., 2006; Adams & Cory, 2007).

The Bcl-2 family proteins function as a "life/death switch" that determines whether or not cell death pathways are activated dependent upon intercellular and intracellular cues. The switch operates by the interaction between the anti-apoptotic proteins and pro-apoptotic proteins. The BH3-only proteins function as damage sensors, and they are activated in response to the cellular stress and DNA damage. BH3-only proteins initiate cell death through activating Bax and Bak by binding with Bax and Bak to induce their oligomerization; alternatively, they engage the anti-apoptotic proteins to prevent them from countering Bax and Bak activation. In normal conditions, BH3-only proteins are held inactive by anti-apoptotic proteins (Letai et al., 2002; Certo et al., 2006; Walensky et al., 2006; Adams & Cory 2007). For example, the anti-apoptotic proteins, Mcl-1 and Bcl-xL, sequester an active form of Bak at the mitochondrial membrane under normal situations. In response to cytotoxic stimuli, activated BH3-only proteins can displace Bak from both Mcl-1 and Bcl-xL, and Bak is activated (Chen et al., 2005; Willis et al., 2005; Willis et al., 2007). After activation, Bax and Bak can induce the MOMP and the intermembrane space components are released as described above.

The activity of Bcl-2 family proteins is regulated at different levels and by different mechanisms, including transcriptional control, posttranslational modification and turnover. For example, Bcl-2 levels may be controlled by micro-RNAs, such as miR-15a and miR-16-1, at transcriptional level since Bcl-2 protein is overexpressed by malignant B cells in chronic lymphocytic leukemia (in which miR-15a and miR-16-1 are deleted or down-regulated -- Cimmino et al., 2005). The activity of bcl-2 proteins also can be affected by phosphorylation at a posttranslational level (Deng et al., 2004). Mcl-1 particularly is labeled by phosphorylation and proteasomally degraded early in response

to many cytotoxic signals (Cuconati et al., 2003; Nijihawan et al., 2003). Noxa and Puma are under the p53-mediated transcriptional regulation and are upregulated upon DNA damage (Nakano & Vousden 2001; Oda et al., 2000). Growth factor deprivation induces the dephosphorylation and activation of Bad (Wang et al., 1995).

In addition to caspases and bcl-2 family proteins, other proteases, such as cathepsins and calpain are also related to apoptotic cell death and involved in the regulation of the activity of caspases and mitochondrial functions.

**Cathepsins** Cathepsins are the major lysosomal proteases. Based on the amino acid comprising the active site, all cathepsins are subdivided into three distinct groups: 1) serine proteases: cathepsins A and G; 2) cysteine proteases: cathepsins B, C, H, K, L, S, and T; 3) aspartate proteases: cathepsins D and E (Turk et al., 2001). Like the caspases, cathepsins are synthesized as inactive precursors, here preproenzymes that first become procathepsins in endoplasmic reticulum and then undergo proteolytic processing to become active and mature enzymes in the acidic environment of late endosome or lysosome (Fujita et al., 1991).

In response to a wide variety of death stimuli, lysosomal cathepsins, especially the cysteinyl protease cathepsin B and L and aspartyl protease cathepsin D, can kill cells, especially by apoptosis (Fehrenbacher et al., 2004). Cathepsin B is essential for many models of apoptosis, including bile-induced hepatocyte apoptosis (Canbay et al., 2003), TNF-α-induced apoptosis in primary hepatocytes and tumor cells (Foghsgaard et al., 2001; Guicciardi et al., 2001), and neuronal apoptosis in PC12 cells after serum deprivation (Shibata et al., 1998). Fibroblasts isolated from cathepsin D-deficient mice

are more resistant to cell death induced by stress (doxorubicin, etoposide and α-tocopheryl succinate) than their normal counterparts (Wu et al., 1998; Neuzil et al., 2002); HeLa cells with cathepsin D antisense constructs show inhibition of IFN-γ and Fas-induced cell death (Deiss et al., 1996); and PC12 cells overexpressing cathepsin D die more than wild type cells following serum deprivation (Shibata et al., 1998). Thus, cathepsin D acts as a mediator of cell death (Tardy et al., 2006). Cathepsin D is implicated in apoptosis induced by staurosporine (Bidere et al., 2003), oxidative stress (Kagedal et al., 2001), and TNF-α (Demoz et al., 2002). Cathepsin L is involved in β-amyloid-induced apoptosis in rat cortical neurons and ultraviolet-mediated apoptosis in human keratinocytes (Welss et al., 2003).

Cathepsins can translocate from the lysosome lumen to the cytosol in response to a variety of death stimuli after lysosome leakage is mediated by sphingosine or ceramide (Heinrich et al., 1999; Gowran & Campbell 2008). Once cathepsins are released into the cytosol, they can directly cleave the cellular substrates or initiate the destabilization of the mitochondria and the activation of caspases (Bidere et al., 2003; Liu et al., 2003; Guicciardi et al., 2004; Li et al., 2007). Mitochondrial permeability transition (MPT) can be induced by lysosomal enzymes via proteolytic activation of phospholipases or bcl-2 family members, such as Bid, Bax and Bak. Lysosomal cysteine proteases, including cathepsin B, H, and L, may be able to cleave Bid, resulting in the activation of Bax and/or Bak, and then pro-apoptotic mitochondrial factors such as cytochome c, AIF and Smac/DIBLO can be released into the cytosol (Dietrich et al., 2004; Cirman e tal., 2004; Houseweart et al., 2003; Heinrich et al., 2004; Terman et al., 2006). The lysosomal enzymes are also able to cleave and activate caspases directly. For example, cathepsin B

can process procaspase-1 and –11 *in vitro*. However, both caspase-1 and –11 are mainly involved in inflammatory response not in apoptosis (Schotte et al., 1998; Vancompernolle et al., 1998; Guicciardi et al., 2004). Cathepsin L can induce activation of procaspase-3/-7 in a cell-free system and cell culture (Kitareewan et al., 2007). Cathepsin L also activates procaspase-3 indirectly through the activation of a un-identified membrane-bound protease in the lysosome (Katunuma et al., 2001).

In summary, cathepsins are involved in the initiation and regulation of cell death by relating to the activity of other death related genes, although their roles are still unclear. In this thesis, we will explore the activity and regulation of Cdk5 in cathepsin B, D, or L null cells to find out how Cdk5 activity fits into the lysosomal cell death pathway and how cathepsins affect cell death.

**Calpain** Calpains, first discovered by Guroff in the CNS in 1964 (Guroff, 1964), are cysteine proteases that reside in the cytosol as zymogens (Johnson, 2000). At least 15 calpains have been identified, of which two major isoforms named μ-calpain (calpain I) and m-calpain (calpain II) are widely distributed in mammalian tissues (Yadavalli et al., 2004; Yang et al., 2004). Elevation of intracellular calcium concentration, proteolytic cleavage and association with membrane phospholipids are required for the activation of calpains (Camins et al., 2007). The activity of calpain is regulated by calpastatin, which is the specific endogenous calpain inhibitor. In the ER, the catalytic and regulatory subunits are associated with calpastatin. A high concentration of calcium activates calpain by causing calpastatin to dissociate from calpain heterodimer and membrane phospholipids (Coolican & Hathaway, 1984;; Hood et al., 2004; Suzuki et al., 2004).

After calpain is activated, it can cleave a variety of substrates, including cytoskeleton proteins, growth factor receptors, mitochondria, actin-binding proteins (spectrin, actin, gephyrin, ankyrin, talin), tubulin, microtubule-associated proteins (Map2, tau), and neurofilaments (Yang et al., 2004; Camins et al., 2007). Calpain plays a role in apoptosis (Liu et al., 2005). Calpain activation was first found in apoptotic cell death in thymocytes (Squier et al., 1994). Calpain shares many substrates with caspases (Casiano et al., 1998) and calpain activates proapoptotic proteins, such as caspase-3, caspase-12 and c-jun N-terminal kinase (JNK), suggesting that calpain activation is involved in apoptosis. Cross-talk between calpain and caspases has been reported during neuronal apoptosis induced by a prion protein fragment (O'Donovan et al., 2001). Calpain activation also leads to the rupture of the membrane of lysosome and the subsequent release and activation of lysosomal cathepsins, culminating in the autolytic digestion of the cell (Leist & Jaattela, 2001; Yamashima, 2004). Yamashima suggested a possible relationship between these three proteases: calpain induces cathepsin release; cathepsin mediates caspase activation, which is involved in calpastatin degradation, thereby facilitating calpain activity (Yamashima 2000; Camins et al., 2006; Raynaud & Marcilhac 2006).

Calpain activity also regulates Cdk5 activation during cell death since the activator of Cdk5, p25, during cell death is formed from the cleavage of p35 by calpain (Tsai et al., 2004). In this thesis, we will evaluate whether Cdk5 activation requires calpain activity during cell death.

**Extrinsic pathway of apoptotic cell death** The extrinsic pathway is activated when the death receptors (DRs) Fas ligand or TNF-α (tumor necrosis factor α) bind with their

receptors to form a receptosome complex. These receptors are characterized by extracellular cysteine-rich domains (CRD) and intracellular death domains (DDs) (Ashe & Berry, 2003; Wesche –Soldato et al., 2007). The extracellular CRD are responsible for the receptor-ligand interaction and receptor self-association (Siegel et al, 2000). The intracellular DDs interact with other DD containing proteins functioning as adaptors, such as TNF (tumor necrosis factor)-RI-associated protein death domain (TRADD), Fas-associated protein with death domain (FADD), receptor-interacting protein (RIP) and RIP-associated Ich-1/CED3 homologous protein with DD (RAIDD) (Aravind et al, 1999; Hofmann 1999; Takeda et al., 2007) in the signal transduction cascade and then the adaptor proteins can interact with a variety of other proteins to complete the signaling pathways (Denecker et al 2001;Ashe & Berry, 2003). Death receptors can bind with the initiator procaspase-8 and –2 via adaptor to form the death-inducing signaling complex (DISC) (Salvesen & Dixit, 1999; Wajant, 2002; Kumar, 2007), which can either activate the downstream effector procaspases (-3, -6 and –7) or activate caspase-8, which in turn mediates the cleavage of Bid (a proapoptotic member of bcl-2 family) to induce the release of cytochrome c and promote the release of Smac/DIABLO (secondary mitochondrial activator of caspases/direct IAP-binding protein of low isoelectric point) (Gross et al, 1999; Madesh et al, 2002; Kim et al., 2006). Caspase-2, similar to caspase-8, can induce the release of cytochrome c, Smac/DIABLO, and AIF (apoptosis-inducing factor) through the cleavage of Bid directly by caspase-2 (Guo et al, 2002; Le Bras et al., 2006), then complete the death pathway (discussed in the next paragraph).

**Intrinsic pathway of cell death** The intrinsic pathway is triggered by a number of factors, such as environmental insults, senescence and developmental programs. The mitochondria are the primary intracellular initiation sites of this pathway and the Bcl-2 family proteins are the central players in the regulation of this pathway; this pathway is therefore also referred to as the mitochondrial cell death pathway (Wright et al., 2004). Mitochondrial outer membrane permeabilization requires the pro-death mitochondrial bcl-2 family proteins, Bax and Bak (Cheng et al., 2006). The opening of the mitochondria permeability transition (MPT) pore results in a loss of mitochondrial membrane potential (MMP) and the release of pro-apoptotic proteins from mitochondria, such as cytochrome c, Smac/DIABLO, Omi/Htr2A and AIF (Sun et al, 2002; Ashe & Berry, 2003; Polster et al., 2005). Cytochrome c released into cytosol interacts with Apaf-1 (apoptotic-activating factor-1), ATP/dATP and caspase-9 to form the apoptosome complex, and then activates caspase-9 (Gogvadze & Orrenius, 2006; Yu et al., 2006). The activated procaspase-9 proteolytically activates procaspase-3 and –7, which results in cell death through controlled proteolytic processing of downstream targets. Responding to the apoptotic stimuli, Smac/DLABLO is released to bind with the inhibitor of apoptosis protein (IAP) and remove its inhibition of caspase activity (Srinivasula et al, 2000; Kumar, 2007). The third mitochondrial factor, AIF, is released from mitochondria and translocated into the nucleus to induce partial DNA fragmentation and chromatin condensation (Ashe & Berry, 2003; Krantic et al., 2007).

The two cell death pathways are largely independent. However, cross-talk and integration between the mitochondria and the extrinsic pathway is provided by Bid, a pro-apoptotic member of Bcl-2 family that can activate caspases. Caspase activation and

mitochondria can respond to both extracellular death stimuli and internal insults. The extrinsic and intrinsic death pathways converge on the mitochondria through activation of pro-apoptotic members of the bcl-2 family (Hengartner 2000; Le Bras et al., 2006).

In summary, apoptosis plays important roles in many aspects, including development, homeostasis and pathogenesis. It involves a series of biochemical events leading to a characteristic cell morphological and death, and it is tightly regulated by diverse genes, such as caspases, bcl-2 family, cathepsins and calpains. In this thesis, we will explore the involvement of Cdk5 activity in aoptosis and the relationship between Cdk5 and these apoptosis related genes.

**2. Autophagic cell death**

Autophagy is a well-known physiological mechanism by which cytoplasmic components are degraded to serve as nutrients for cell survival during nutrient deprivation. Additionally, autophagy is considered as a housekeeping mechanism to maintain homeostasis by eliminating excessive or unnecessary proteins and injured or aged organelles in normal cells (Sluijters et al., 2000; Ogier-Denis & Codogno 2003; Lockshin & Zakeri 2004). A number of studies indicate that autophagy plays a dual role in modulating cell viability and cell death. Autophagic cell death is characterized by the accumulation in the cell of the acidic autophagic vacuoles accompanied by organelle morphological changes, such as nuclear condensation and massive cellular degeneration (Schweichel & Merker, 1973; Thummel, 2001; Ogier-Denis & Codogno, 2003). One of the key differences between apoptosis and autophagy is that the cells that die through

apoptosis are degraded by lysosomes of a second, phagocytic cell and cells that die through autophagic cell death are destroyed by its own lysosomes (Thummel, 2001).

Autophagy is recognized as a protein degradation system in which the proteins are delivered to the lysosome and are digested by lysosomal hydrolases (Kondo & Kondo, 2006). At least three different autophagic pathways have been described, such as macroautophagy, microautophagy and chaperone-mediated autophagy. Among the three pathways, macroautophagy is the most universal degradation pathway. During macroautophagy, portions of the cytoplasm and various organelles such as endoplasmic reticulum, mitochondria and peroxisomes are sequestered within double membrane vacuoles called autophagosomes that can fuse with lysosomal compartments, and finally the materials are degraded in autophagolysosomes (Dunn, 1994; Cuervo, 2004; Terman et al., 2006). In microautophagy, the cytoplasmic components enter lysosomes directly through invagination of their membrane. In chaperone-mediated autophagy, target proteins with a specific motif (KFERQ) are recognized and delivered to the lysosomes by chaperones (Majeski & Dice 2004; Tardy et al., 2006).

Autophagy can occur in caspase-dependent or caspase-independent manners, and many genes are involved in the regulation of autophagic cell death. These autophagy-related proteins including Atg proteins, beclin 1, phosphatidylinositol 3-phosphate kinase (PI3K) and microtubule-associated protein 1 light chain 3 (LC3) are involved in the regulation of the initiationand maturation of autophagy in the absence of nutrients and in the response to other stimuli (Levine & Deretic 2007). Atgs, genes that are involved in autophagic cell death, were first identified in genetic studies of autophagy in yeast. The Atg genes were identified by the study of autophagy induced by nutrient starvation in

yeast, and they are required for the activation of the signaling complex that triggers the formation of autophagosome (Harding et al., 1995; Klionsky et al., 2003). Atg7 and Atg5 were identified as autophagy modulators (Baehrecke, 2005, Bredesen et al., 2006). Atg5 is required for vacuole formation during cell death induced by IFN-γ in Hela cells (Pyo et al., 2005). Atg1 and a complex of PI3K and beclin 1 lead to activation of downstream Atg factors by initiating the conjugation of Atg 12 with Atg 5 and 16, and triggering the oligomerization on the outer membrane of the growing autophagosome. LC3 is part of the conjugation system and undergoes the conversion from the unmodified LC3-I to LC3-II, which is associated with both inside and outside membrane of autophagosome. LC3-II has been used as a marker of autophagy (Deretic 2005; Kabeya et al., 2000; Kondo & Kondo 2006). Atg genes are conserved from yeast to human. Beclin 1 (Atg 6) is a mammalian autophagic gene that induces autophagy in cultured breast cancer cells, MCF-7 (Liang et al., 1999). Beclin 1 is a Bcl-2/Bcl-xL interacting protein; the loss of interaction between beclin 1 and bcl-2 leads to an autophagy gene-dependent cell death (Tardy et al., 2006). PI3K is also related to autophagy. In mammalian cells, the formation of autophagosome is dependent on the activity of PI3K, since the inhibition of PI3K by 3-methyl-adenine (3-MA) blocks the formation of autophagosomes (Seglen & Gordon, 1982). In addition to these autophagy-related genes, apoptotic genes are also associated with autophagy. For example, autophagic cell death can be induced through pro-apoptotic proteins and antiapoptotic protein, Bcl-2, can also inhibit autophagy (Saeki et al., 2000; Pattingre et al., 2005; Pyo et al., 2005).

### 3. Necrotic cell death

The prominent features of necrosis are an electron-lucent cytoplasm, swelling and breakdown of organelles, and rapid plasma membrane rupture with few nuclear changes (Kerr et al., 1972; Wyllie et al., 1984; Kitanaka & Kuchino 1999, Malhi et al., 2006). These morphological changes result from the inability of the cell to maintain the electrochemical potential due to increased mitochondrial ROS (reactive oxygen species) production, channel-mediated calcium uptake, activation of nonapoptotic proteases, or enzymatic destruction of cofactors required for ATP production (Zong & Thompson 2006). Although the mechanisms of necrosis in development and homoeostasis are still unclear, necrosis may also be involved in elimination of unwanted cells during development. The dead cells in the interdigital area in apaf-1 deficient embryos appear to be necrotic (Chautan et al., 1999). In caspase-3 deficient embryos necrotic cell death is also involved in inner ear development (Raphael 2002).

Traditionally, necrosis was considered as an uncontrolled, passive, energy-independent process. However, more recent studies have suggested that necrotic cell death may be a regulated process in which signal transduction and metabolic pathways are involved (Vandenabeele et al., 2006). RIP1(receptor interacting protein 1), death domain-containing serine and threonine kinase are important for necrosis and PARP (poly(ADP)-ribose polymerase, a DNA damage repair enzyme), which is cleaved by caspases is activated during the induction of necrotic cell death (Ha & Snyder, 1999; Zong et al., 2004; Vandenabeele et al., 2006).

Many proteases are implicated in necrosis, including calpains (Liu et al., 2005; Kagedal et al., 2001). Calpain activity is involved in hypoxia-induced necrotic cell death, and hypoxia-induced cell death switches to apoptotic cell death when calpain activity is

inhibited (Kim et al., 2007). Calpain is also involved in glucose-induced necrosis, since the inhibition of calpain activity is able to reduce the degree of early high glucose-induced necrosis (Harwood et al., 2007).

Although necrosis is considered as a distinct and independent phenomenon from apoptosis, an alternative view is that necrosis and apoptosis are interdependent phenomena resulting from activation of shared pathways and signals (Lemasters, 1999). In cultured hepatocytes when necrosis is blocked by fructose plus glycine, apoptosis occurs instead. Otherwise when the apoptotic signaling pathway is inhibited in the absence of ATP, necrotic cell killing pathway is activated while simultaneously apoptosis is suppressed (Malhi et al., 2006).

In this thesis, we will deregulate some of apoptosis related genes, such as caspase-3, cathepsins and calpain to examine whether the type of cell death is altered in these situations.

**4. Other types of cell death**

Although most cell death follows one of the three major types as mentioned before, not all deaths fit into the morphological and biochemical characteristics of these three categories. There are situations in which cells die without using one of these pathways or using parts of the different pathways, resulting in a type of cell death that is like none of the three types mentioned above. These types of cell death may include variations in the degree of chromatin condensation and margination or the extent of blebbing (Lockshin & Zakeri 2002), such as apoptosis-like and necrosis-like PCD. For example, in caspase-3 deficient MCF-7 cells, apoptosis-like PCD can be induced by the

depletion of Hsp70, resulting in an incomplete chromatin condensation (Nylandsted et al., 2000). In necrosis-like PCD, no chromatin condensation exists, as is seen in the specialized caspase-independent cell death signaling pathways (Leist & Jaattela, 2001; Kajstura et al., 2006). Necrosis-like PCD can be induced by the inhibition of PARP activation (Ha et al., 1999; Ohno et al., 2008), mutation of the intracellular signaling molecules (Holler et al., 2000), and oxygen-radical scavengers (Vercammen et al., 1998; Valencia & Moran 2004).

Notably, different types of cell death can coexist during cell death progression. Many toxins induce apoptosis at low concentration and necrosis at high concentration. At the late stage of apoptosis, necrotic features appear due to the loss of the plasma membrane integrity and the depletion of the cellular energy (Majno & Joris 1995). Injured cells undergo both apoptosis and necrosis during brain ischemia (Beilharz et al., 1995; Wang et al., 2007) or liver damage induced by death domain receptor ligands (Leist et al., 1995; Ouasti et al., 2007).

Identification of the different types of cell death at a general biological level and understanding the mechanisms of cell death can help us to control cell death in diseases. In this thesis, we will evaluate the mode of cell death by using different biochemical and morphological characteristics in mouse embryonic fibroblast cells exposed to death stimuli when the activities of genes such as Cdk5 or cathepsins are de-regulated. We will also answer whether the deregulation of Cdk5 activity affects the mode of cell death and whether the lack of cathepsin B, D, or L alters the type of cell death induced by different death stimuli.

## Chapter III. Cyclin-dependent kinase 5 (Cdk5)

1. Structure of Cdk5
2. Regulation of Cdk5
3. Activity of Cdk5

Cell cycle and cell death are two separate but highly related events during the life of a cell. However, cell death can result from the deregulation of cell cycle and it can occur at any phase of the cell cycle (Ucker, 1991; Rubin et al., 1992). Cyclin-dependent kinases (Cdks) are the master regulators of cell cycle. The first identified Cdk, Cdc2, is essential for both G1/S and G2/M transitions in the *S. pombe* cell cycle (Nurse, 2000). Cdc2 homologues were subsequently found in all eukaryotes; in humans. 11 Cdks have been identified (Malumbre & Barbacid, 2005). Cdks are proline-directed serine/threonine protein kinases; the periodic activation of Cdks by their activators, called cyclins, regulates eukaryotic cell cycle. For example, Cdk2 can interact with cyclin E to induce the initiation of DNA synthesis at the beginning of S phase, then bind to cyclin A through S phase, and the Cdk1-cyclin B complex initiates mitosis (Nebreda, 2006). In addition, cyclin D-Cdk4/6 complexes are also involved in the regulation of G1/S progression, while cyclin B-Cdc2 complex mediates M-phase progression (Ekholm & Reed, 2000; Pines 1995). On the other hand, apoptotic stimuli can induce the activation of certain Cdk-cyclin complexes and then cell death results from the disturbance of the progress of the normal cell cycle. Cdk2 and Cdk6 are activated in embryonic cortical neuronal death induced by the DNA-damage agent, camptothecin (Ghahremani et al., 2002; O'Hare et al., 2005). The inappropriate activation of cell cycle regulators including Cdks, and cyclins may control ischemic neuronal cell death. In different models of ischemia in vivo, kinase activity of Cdk4/6 and their activator cyclin D1 are upregulated (Rashidian et al., 2007). Cyclin D1 is expressed in the infarct region in MCAO (middle cerebral artery occlusion) model of focal ischemia (Katchonav et al., 2001; Wen et al., 2005) and aberrant Cdk4 expression has been shown in the MCAO model (Hayashi et al., 1999).

Cdk5 is also involved in a process other than cell cycle -- cell death. Cyclin-dependent kinase 5 (Cdk5) is considered a member of the Cdk family due to its sequence homology to the prototypic Cdk, human Cdc2. It is also known as neuronal Cdc-2-like kinase (Nclk) (Lew et la., 1992). However, Cdk5 is a unique member of the family of Cdks, because its major activators are not cyclins and its activity is not relevant to the cell cycle progression.

**1. Structure of Cdk5**

The sequence of Cdk5 is homologous to the other Cdks, especially the residues forming the ATP pocket (Sridhar et al., 2006). Cdk5 is highly conserved in evolution and it shares 99% identity at protein level among vertebrates. Like other Cdks, Cdk5 is a proline-directed kinase that phosphorylates serine or threonine immediately N-terminal of a proline site. The sequence, (S/T)PX(K/H/R), is the conserved motif in its substrates (Maccioni et al., 2001). Structurally, Cdk5 forms a bilobal 3D-structure that is a homology model of Cdks and is typical in most protein kinases. The small lobe is N-lobe, which is rich in beta-sheets and contains the glycine-rich motif for ATP binding. The PSSALRE helix in this lobe is important for the interaction with subunits, which is a common feature in all Cdks. Lys33, Glu51 and Asp143 are three conserved residues forming a catalytic triad that helps orient ATP and facilitate catalysis. The bigger lobe is C-lobe, which contains a T-loop which can move down from the inhibitory position to a fully relaxed configuration, allowing the association of regulatory subunits, which permits the binding of the substrates for phosphorylation subsequently (Sharma et al., 1999).

**2. Regulation of Cdk5**

As for other Cdks, monomeric Cdk5 is enzymatically inactive and the activation of Cdk5 requires association with its specific activators, including p23 (Ishiguro et al., 1994), cyclin E (Donnellan et al., 1999), p35 (Tsai et al., 1994), p25 (Ishiguro et al., 1994), p39 (Tang et al., 1995), p67 (Shetty et al., 1995), and RINGO (Dinarina et al., 2005). Among all the listed activators, p35 and p39 are two major neuronal Cdk5 activators, associated with cell differentiation and cell death. The functions of other activators remain unclear; however some investigators have suggested that they are associated with Cdk5 activity. The complex of p23 and Cdk5 is able to phosphorylate Tau protein (Lew et al., 1992) and may play a role in regulation of cytoskeleton dynamics (Michel et al., 1998). Cyclin E is also found in the nervous system and it is possible that the interaction of Cdk5 and cyclin E is related to the control of cell cycle (Donnellan et al., 1999; Verdaguer et al., 2004). P67 also increases Cdk5 kinase activity in vivo and Cdk5/p67 may participate in the phosphorylation of cytoskeleton proteins (Shetty et al., 1995; Rajgopal et al., 2001; Sharma et al., 2005).

The primary sequences of p35 and p39 display no significant homology to cyclins, although their tertiary structure is assumed cyclin-like. p35 and p39 can be cleaved by a calcium dependent protease, calpain, to generate p25 and p29, which also have cyclin-like structure (Tsai et al., 2004). The cleavage of the N-terminal of p35 and p39 produces C-terminal truncated proteins p25 and p29 that retain the ability to bind with Cdk5 and activate Cdk5. When Cdk5 binds with p25 and p29, the conformation of complexes is indistinguishable from active Cdk2 (Mapelli & Musacchio 2003; Zhang et al., 2007). Further, the p25 and p29 have an extended half-life (5-10-fold greater than p35

and p39), which leads to the prolonged activation of Cdk5 (Tsai et al., 2004; Kusakawa et al., 2000; Patzke & Tsai, 2002). In addition to the difference of stability between p35/p39 and p25/p29, the Cdk5-p25 complex is different from Cdk5-p35 complex with regard to the cellular localization, kinase activity, and substrate selection, which are involved in the regulation of Cdk5 activity. For example, it has been suggested that the N-terminal region of p35 determines the activator-specific cellular localization, turnover rate, or substrate specificity of the complexes. The kinase activity of Cdk5/p39 complex was decreased after sodium chloride treatment, while that of Cdk5/p39ΔN was not. A similar N-terminally deleted form of p35, p35ΔN, can form a stable complex with Cdk5 in response to detergent (Yamada et al., 2007). The target cellular location of p35 and p39 is the cell membrane because of their N-terminal myristoylation signals, which limit the active Cdk5 to membrane structures. P35 is in particles and p25 is soluble, which indicates that p35 and p25 have different localization (Patrick et al., 1999; Kusakawa et al., 2000). The cleavage of p35 and p39 changes the subcellular distribution of active Cdk5 from membrane to the cytosolic fraction, which may result in the alteration of the substrate specificity of Cdk5 and different localization of Cdk5 activity (Cheung et al., 2004; Rademakers et al., 2005; Kamei et al., 2007). It has also been found that Cdk5-p25 complex can be transported into the nucleus from cytosol during cell death. The accumulation of Cdk5-p25 in the nucleus was reported in neurons induced into apoptosis by camptothecin (O'Hare et al., 2005; Saito et al., 2007), which suggested that Cdk5-p25 may target the transcriptional factors or nuclear proteins in the nucleus. For example, Cdk5-p25 is able to inactivate the survival transcriptional factor, MEF2 by phosphorylation after it is translocated into nucleus, or it may activate cell cycle

machinery that is inactive in differentiating neurons by phosphorylating nuclear proteins, such as pRb and p53 (Lee et al., 1997; Zhang et al., 2002; Gong et al., 2003).

Cdk5 activity may also be regulated by phosphorylation. First, Cdk5 protein itself can be phosphorylated at three sites: Ser159, Thr14, and Tyr15, and the phosphorylation of Cdk5 at different sites correlates with the regulation of Cdk5 activity. Phosphorylation of Ser159 increases the activity of Cdk5, and adding a phosphate group on Ser159 can affect the association of Cdk5 with its activators (Sharma et la., 1999; Tarricone et al., 2001). Thr14 can be phosphorylated by a protein kinase purified from bovine thymus cytosol; this phosphorylation inhibits the activation of Cdk5 (Matsuura et al., 1996). However, the phosphorylation of Tyr15 mediated by c-Ab1 can stimulate p35-mediated Cdk5 activation (Zukerberg et al., 2000). Furthermore, the activator of Cdk5, such as p35, can also be phosphorylated, affecting the susceptibility of p35 to calpain cleavage; phosphorylated p35 is not cleaved by calpain (Saito et al., 2007). Autophosphorylation of p35 by Cdk5 at Ser8 and Thr138 suppresses p35 cleavage to p25 by calpain, which increases and prolongs the Cdk5-p35 activity during development and decreases the production of p25 (Saito et al., 2003; Kamei et al., 2007).

The activity of Cdk5 also can be regulated by other proteins, such as caspase-3 and TNFα. The production of p25 from the cleavage of p35 during cell death is caspase-3 dependent, because active caspase-3 digests the endogenous calpain inhibitor calpastatin, and thus promotes the generation of p25 (O'Hare et al., 2005). As mentioned above, the production of p25, which has longer half-life and is more soluble in cytosol, prolongs and relocalizes Cdk5 activity during cell death. Orenllana et al. have suggested that TNFα induces a decrease of Cdk5 activity in rat hippocampal neurons and has no effect on the

level of p35, which indicates that TNFα can modulate Cdk5 activity at protein level directly without affecting its activator (Orenllana et al., 2007). A nuclear protein, SET, interacts with Cdk5/p35 to upregulate its kinase activity (Qu et al., 2002).

These several results indicate that Cdk5 activity is tightly regulated by different means. But it appears that the availability of its activators and the regulation of its stability, as well as cellular distribution and activity of its activators are the major and rate-limiting steps in the Cdk5 activation.

### 3. Activity of Cdk5

As demonstrated by the fact that Cdk5 is an essential molecule in the brain and its activity is primarily associated with neurons. However, more recent studies have demonstrated that the kinase activity of Cdk5 is more general, and that Cdk5 activity is present in many different cell types and is involved in different processes such as differentiation (Miyamoto et al., 2007), exocytosis (Xin et al., 2004), gene expression (Choe et al., 2007), cell migration (Feng & Walsh 2001), tissue regeneration, wound healing and apoptosis (Cruz & Tsai 2004; Guo, 2006). The multifunctional roles of Cdk5 are completed by virtue of phosphorylating diverse substrates, including cytoskeletal elements (Causeret et al., 2007), cell adhesion molecules (Nakano et al., 2005), signal transduction kinases in cytoskeletal regulation or neuronal migration, and proteins in membrane cycling, axon transportation and synaptic plasticity (Feng & Walsh, 2001; Smith et al., 2001 Guo, 2006).

For some functions, the relevant target substrates have been identified, especially tau, neurofilaments, MAPs (mitogen-activated proteins), and the elements in the axonal

cytoskeleton (Grant et al., 2001). For example, the hyperphosphorylation of tau protein mediated by Cdk5 results in a reduction of its affinity for microtubules and ability to stabilize microtubules to promote the development of the neurofibrillary tangles (NFTs) that is a hallmark of neurodegenerative diseases (Noble et al., 2003; Zheng et al., 2005). In addition, Cdk5 can phosphorylate JNK3 to inhibit JNK3 from playing a role in neuronal survival (Li et al., 2002); the intermediate filament protein nestin to regulate the development of neuronal and myogenic tissues (Sahlgren et al., 2003); the NMDA receptor and P/Q type voltage dependent calcium channel (VDCC) to control calcium influx and regulate neuronal transmitter release (Kerokoski et al., 2004; Pareek et al., 2006); huntingtin, which is an antiapoptotic protein, to protect it from cleavage by caspases and regulate its toxicity (Luo et al., 2005); and parkin to inhibit its ubiquitinylation and degradation, which results in the accumulation of toxic parkin in Parkinson disease (Avraham et al., 2007). Several transcriptional factors were also identified as substrates of Cdk5 in the nucleus, including myocyte enhancing factor-2 (MEF-2) and transducer and activator of transcription 3 (STAT3), for which phosphorylation modulates its transcriptional activity in muscle (Fu et al., 2004; Gong et al., 2003). STAT3 pathway was involved in Cdk5-dependent proliferation of medullary thyroid carcinoma (MTC) cells through phosphorylation. In addition, Cdk5 inhibition reduced nuclear distributions of both the Cdk5-p35 complex and phospho-STAT3 in MTC cells, accompanying the reduction of phospho-STAT3 (Lin et al., 2007).

**1). Activity of Cdk5 in Cell Differentiation**

Although Cdk5 activity is found in many situations, its kinase activity has been most examined in the development of the central nervous system (CNS). Cdk5 activity

plays a role in neurite migration (Hirota et al., 2007), axon patterning (Connell-Crowley et al., 2000), cortical lamination (Rakic et al., 2006), neuronal secretion (Rosales et al., 2004), neuronal adhesion (Homayouni & Curran 2000), differentiation of oligodendrocytes (Miyamoto et al., 2007), formation of synaptic structure and plasticity and the maintenance of neuronal cytoarchitecture (Howasli & Bibb 2007). Cdk5 null mutant mice as well as p35/p39 double null mutants mice die perinatally and display widespread defects in migration of many brain compartments including cerebral cortex, hippocampus and cerebellum, and exhibit a reverse of normal cortical laminal architecture, cytoarchitectural disturbance in the cerebellum, brainstem and hippocampus (Ko et al., 2001; Angelo et al.,2006). The induction of p35 expression and the increase of Cdk5 activity are involved in the differentiation of PC12 cells by NGF (Harada et al., 2001). Cdk5 helps maintain the stability of the neuronal cytoskeletal network by stabilizing neurofilament proteins in neurons, and it also modulates neurofilament metabolism in axon outgrowth and radial growth (Grant et al., 2001).

Recent investigations have focused on the implication of Cdk5 activity in the connectivity of developing neurons. In addition to the involvement of Cdk5 in axon guidance via its effect on growth cone collapse and neurite actin dynamic, Cdk5 has been associated with synaptogenesis including the formation of synaptic structures and the synaptic vesicle cycles through the phosphorylation of synapsin I, Munc18 and VDCC (Cheung et al., 2006; Fu et al., 2005). Phosphorylation of tyrosine hydroxylase by Cdk5 is the rate-limiting step in the synthesis of catecholamines including dopamine, which suggests that Cdk5 activity is involved in the regulation of neurotransmission (Kansy et al., 2004). Although Cdk5 plays roles predominantly in the CNS, recent reports about its

new role in pain signaling indicates its activity in the peripheral nervous system (PNS). Cdk5 and p35 are co-expressed in dorsal root ganglia, trigeminal ganglia and spinal cord, and Cdk5 activity is increased in a response to peripheral inflammation (Pareek et al., 2006; Pareek & Kulkarni 2006).

Cdk5 activity is also present in non-neuronal cells. Cdk5 functions in myogenesis. Cdk5 expression and activity are increased during early myogenesis in murine C2 cells and Cdk5 activity may be required for the expression of muscle differentiation markers such as myogenin and troponin T (Lazaro et al., 1997). Dominant negative Cdk5 disrupts muscle patterning and inhibits the expression of the MyoD and MRF4 regulators of myogenesis (Philpott et al., 1997). Cdk5/p35 also can act as a regulator of monocyte differentiation. In human myeloid leukemia HL60 cells, Cdk5 associating with p35 results in the expression of CD14 and nonspecific esterase marker of monocytic differentiation (Chen et al., 2001). Cdk5/p35 complex is also a modulator of sperm tail development and differentiation via outer dense fiber (ODF) phosphorylation. Increased Cdk5/p35 activity was observed in the isolated elongating spermatids (Rosales & Lee, 2006). During glomerulogenesis, Cdk5/p35 activity has been detected in differentiating visceral glomerular epithelial cells (podocytes). However, in the mice with anti-glomerular basement membrane nephritis and in the proliferating and differentiated podocytes of HIV transgenic mice, the level of Cdk5 expression and activation was decreased, which resulted in cellular elongation and loss of arborized phenotype (Griffin et al., 2004).

**2). Activity of Cdk5 in cell death**

In addition to its roles in development and differentiation, Cdk5 also play a role in cell death.

In developing mouse nervous system, massive cell death in the dorsal root ganglia and the trigeminal ganglia is accompanied by the increased level of Cdk5 expression and induced Cdk5 activation (Zhang et al., 1997). During cell death induced by removal of NGF in differentiated PC12 cells, Cdk5 expression and activity were increased (Zhang & Johnson, 2000). Increased Cdk5 activity may be involved in the promotion of neuronal cell death. Many neurological diseases, including Alzheimer's disease (AD), Parkinson disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), and stroke, which manifest a definite apoptotic and necrotic neuronal cell death (Weishaupt & Bahr, 2003; Cheung et al., 2006). The deregulation of Cdk5 by the generation of p25 can trigger various events associated with neurodegenerative diseases. For example, the hyperphosphorylation of tau protein by Cdk5 is related to the formation of tau into neurofibrillary tangles. Cdk5 has a role in modulating β-amyloid precursor protein (APP) processing and β-amyloid generation, which are common features of AD, and the expression of p25 alone is sufficient to induce the formation of neurofibrillary tangles in the absence of mutant human tau (Cruz & Tsai, 2004; Hamdane & Buee, 2007). In PD, Cdk5 phosphorylates parkin, which contributes to the aggregation of toxic parkin substrates and decrease in the ability of dopaminergic cells to cope with toxic insults (Avraham et al., 2007). The involvement of Cdk5 in neuronal cell death was also indicated by a new potential nuclear pathway in which Cdk5 was able to phosphorylate MEF2, once it translocated into nucleus, and inhibit its activity. Since MEF2 is necessary

for neuronal survival, its phosphorylation by Cdk5 may promote neuronal cell death (Verdaguer et al., 2005). Cdk5/p25 is a proapoptotic factor in primary cultured cortical neurons when endoplasmic reticulum stress causes the calpain-dependent cleavage of p35 to p25 (Saito et al., 2007).

The association of Cdk5 expression and activation with cell death is not restricted to neurons. Cdk5 is expressed and activated in different systems during cell death. The increase of Cdk5 expression and activation accompanies cell death in the interdigital areas of retinoic acid-treated developing mouse limb (Ahuja et al., 1997) and in developing mouse embryos treated with cyclophosphamide (Zhu et al., 2002), as well as in all other cell deaths seen during normal development (Lin et al., 2006). The correlation of the expression and activation of Cdk5 with cell death is shown not only in embryonic tissues but also in adult tissues. The up-regulation of Cdk5 activation during cell death induced by cyclophosphamide and camptothecin is post-translational (Zhu et al., 2002; Lin et al., 2006). Cdk5 activity is present in mouse ovary and Cdk5 is expressed in oocytes at all stages of follicle development, particularly dying granulosa cells (Lee et al., 2004; Rosales & Lee; 2006). Elevated Cdk5 activity and p35 expression were found in human M059J glioblastoma multiforme (GBM) cell lines when apoptotic cell death was induced by ionizing radiation, which indicated that Cdk5 was involved in the initiation of apoptosis in human GBM cells (Catania et al., 2001). Dominant negative Cdk5 transfection can reduce the level of cell death while wild type Cdk5 transfection increase the level of cell death in heat-shocked human astrocytoma cells (Gao et al., 2001).

Overall, the kinase activity of Cdk5 is involved in many different facets of an organism's cellular activities, including differentiation and death in both neuronal and

non-neuronal cells. The activity of Cdk5 is tightly controlled by several means, such as binding with its regulators, associating with other proteins and phosphorylation. It is suggested that Cdk5 is activated during cell death by association with its activator of p25, which derives from the cleavage of p35 by calpain and that Cdk5 activity relates more to general cell death than to a specific pathway, since Cdk5 can be activated in the absence of p53, Apaf-1, caspase-9 and –3 (Zhu et al., 2002; Lin et al., 2006). To date, the activation of Cdk5 has been linked to cell death. We hypothesized that the activity of Cdk5 may be a requirement for the induction of cell death and that other genes, such as caspase-3 and cathepsins, are possibly related to the regulation of Cdk5 activity during neuronal or non-neuronal cell death. To test this hypothesis, in this thesis, we will investigate whether Cdk5 activity is involved in caspase-3 independent neuronal cell death during development by using caspase-3 null mouse treated with cyclophosphamide and in cell death pathways related to lysosomes by using cathepsin-null cells during cell death induced by cycloheximide and camptothecin. We will also evaluate whether cell death is affected by downregulating Cdk5 activity using calpain inhibitor, Cdk5 inhibitor, and RNA interference.

# PART II. MATERIALS AND METHODS

## Materials and Methods

### a. Materials

#### *Animals*

Caspase-3 knockout mouse embryos

Caspase-3 heterozygous mice (Gift from Dr. Richard Flavell, Yale University) 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 1X PBS (Phosphate-Buffered Saline, 137 mM NaCl, 2.68 mM KCl, 4.3 mM $Na_2HPO_4$, 1.4 mM $KH_2PO_4$, pH 7.4), and the tail was taken for genotyping. The rest of the embryos were fixed and embedded for sectioning as described below.

Cyclophosphamide-treated mouse embryos

Caspase-3 heterozygous mice 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 12.5 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 18-24 hrs after injection, and the embryos were removed.

#### *Tissue, slide preparation and microscopy*

Embryos were washed with PBS, fixed in 4% paraformaldehyde with shaking at 4ºC 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) and frozen in isopropanol/liquid $N_2$. Special care was used in the orientation of the mouse embryo or limb samples during the embedding procedure to make sure the cutting section represented a good map of the tissue. Frozen sections (5μm) were cut at –20°C and placed on VECTORBOND coated slides and stored at -80°C prior to use.

***Antibodies and reagents***

The calpain inhibitor, PD150606 (Cat# 513022), was obtained from CalBiochem (La Jolla, CA). Cycloheximide (CHX, Cat# C7698) and camptothcecin (CPT Cat# C9911) were obtained from Sigma (St. Louis, MO). *CellTracker* fluorescent dyes (Cat# C34552 & C2925) were obtained from Invitrogen. *Saccharomyces cerevisiae* zymosan A BioParticles conjugated with FITC (Cat# Z2841) was obtained from Invitrogen. The rabbit polyclonal IgG antibodies used in this thesis include anti-Cdk5 (C-8, Cat# sc-173) and anti-PARP (H-250 Cat# sc-7150) antibodies from Santa Cruz Biotechnology (Santa Cruz, CA); anti-p35 (C-19, Cat# P9489), anti-LC3-II (Cat# L7543) and anti-cathepsin L (Cat# C2970) antibodies from Sigma (St. Louis, MO); anti-active caspase-7 antibody (Cat#9491s) from Cell Signaling Technology; anti-phophohistone H1 (Cat# 06-597), antibodie from Upstate Biotechnology (Lake Placid, NY). The mouse monoclonal antibody used is anti-active caspase-3 antibody (Cat# 559565) from BD PharMingen (San Diego, CA)

***Cell culture and treatment***

**1. C8 cells**

C8 is a mouse embryonic fibroblast (MEF) cell line transformed with E1A and ras (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, 50 U/ml penicillin and 100 μg/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 50 μM camptothecin (CPT, dissolved in DMSO), for 8 hrs or 18 hrs (our previous studies show that low level of cell death can be induced at 8 hrs-exposure and high level of cell death can be induced at 18 hrs-exposure). To block calpain activation induced by CPT in C8 cells, the synthetic calpain inhibitor 15 μM PD150606 was co-administered with CPT and cells were incubated for 8 hrs or 18 hrs.

**2. Cathepsin-B -/-, -D -/-, - L -/- and Wild Type mouse embryo fibroblast cells**

Mouse embryo fibroblast (MEF) cells obtained from transgenic mouse embryos with single knockout genotypes of cathepsin-B, -D, or -L or wild type (WT) were immortalized with SV40 (Gift from Dr. Marianne Boes, Harvard Medical School). Cells were cultured in DMEM (Dulbecco's Modified Eagle Medium, Gibco BRL Life Technologies, cat# 12800-017) as above. At 80% confluence, cells were treated in DMEM containing 1% FBS with different cytotoxic reagents, such as 100 μg/ml cycloheximide (CHX, dissolved in ethanol), and 50μM camptothecin (CPT, dissolved in DMSO), and cells were collected at different times (8, 24, 48, 72, 96, and 120 hrs).

**3. COS-7 cells**

COS-7 cells were obtained from the American Type Culture Collection (ATCC) and cultured as above. At 80% confluence, cells were treated in DMEM containing 1% FBS with siRNA of Cdk5 transfected into COS-7 cells to block Cdk5 expression and cells were co-administered with CPT 50 μM for 24 hrs and 48 hrs to induce cell death.

## B. Cellular and Molecular Methods

### *DNA fragmentation assay*

DNA fragmentation was detected by using TUNEL POD (Terminal deoxynucleotidyl transferase-mediated dUTP 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% Triton X-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 37º 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* proteins were 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 (antibodies against 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 3x and incubated with ABC reagent for 2 hrs at RT. Sections were washed with PBST 3x before being visualized with DAB, counterstained with methylene blue, and mounted with Permount.

***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 removed by centrifugation at 13,000 rpm for 30 min 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 (100 mM 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 30 min and incubated with different primary antibodies on the shaker overnight at 4º 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***

Equal amounts of lysates from 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 200 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. The phosphorylated histone H1 was detected by western blot analysis described as above, using anti-phospho-histone H1 primary antibody.

***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. 100 μl of well suspended cells was mixed with 100 μl 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 judged non-viable.

***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 16 μg/ml bis-benzimide in PBS for 25 min at RT. Finally, the cells were washed with ice cold PBS once and resuspended in PBS. 20 μL of the cell suspension was evenly distributed on a slide and coverslipped to be viewed under an Eclipse TE300 microscope (Nikon Inc., Melville, NY).

***PCR genotyping of caspase-3 knockout mice***

Three primers were used: 5'- TGC TAA AGC GCA TGC TCC AGA CTG -3'; 5'- GGG AAA CCA ACA GTA GTC AGT CCT -3'; and 5'- GCG AGT GAG AAT GTG CAT AAA CCT -3'. Briefly, the tail from the mice was lysed in 170 μl of Lysis Solution (50 mM Tris, pH 8.3, 100 mM NaCl, 5 mM EDTA, 0.8% SDS) and 30 μl of Proteinase K solution (10 mg/ml) at 55°C for 5 hrs or overnight. After centrifuging at 16,000 g for 15min, the supernatant containing DNA was precipitated using the same volume of 100% isopropyl alcohol. The DNA pellet obtained by centrifuging at 13,000 rpm for 15 min was dissolved in autoclaved dd$H_2O$ and stored at -20° C. 10 to 200 ng of the extracted DNA was used for PCR. The PCR assay was 30 cycles (1 cycle= 94° C 30

sec. 55° C 30 sec. 72° C 1 min). The PCR products were separated by gel electrophoresis on a 1.5% agarose gel. Wild type mice gave one band at 320 bp, and caspase-3 knockout mice showed one band at 300 bp.

***RNA interference***

The target sequences were designed by the siRNA target finder in http://www.Ambion.com/techlib/misc/siRNA_finder.html. Five 21 nt target sequences were selected: Cdk5-1: **Target sequence:** AAGAUUGGGGAAGGCACCUAU; Antisense template oligonucleptide (DNA): 5'-AAGATTGGGGAAGGCACCTATCCTGTCTC–3'; Sense template oligonucleptide (DNA): 5'-AAATAGGTGCCTTCCCCAAT CCCTGTCTC – 3'; Cdk5-2: **Target sequence:** AAGGCACCUAUGGAACUGUGU; Antisense template oligonucleptide (DNA): 5'-AAGGCACCTATGGAACTGTGTCCTGTCTC –3'; Sense template oligonucleptide (DNA): 5'-AAACACAGTTCCATAGGTGCCCCTGTCTC – 3'; Cdk5-3: **Target sequence:** AAACCGGGAAACUCAUGAGAU; Antisense template oligonucleptide (DNA): 5'-AAACCGGGAAACTCATGAGATCCTGTCTC –3'; Sense template oligonucleptide (DNA): 5'-AATCTCATGAGTTTCCCGGTCCTGTCTC –3'; Cdk5-4: **Target sequence:** AAACUCAUGAGAUUGUGGCUC; Antisense template oligonucleptide (DNA): 5'-AAACTCATGAGATTGTGGCTCCCTGTCTC –3'Sense template oligonucleptide (DNA): 5'-AAGAGCCACAATCTCATGAGTCCTGTC TC – 3'; Cdk5-5: **Target sequence:** AAGAAAUAUUUCGACAGCUGC; Antisense template oligonucleptide (DNA): 5'-AAGAAATATTTCGACAGCTGCCCTGTCTC –3'; Sense template oligonucleptide (DNA): 5'-AAGCAGCTGTCGAAATATTTCCCTG TCTC – 3'.siRNAs were prepared by using *Silence siRNA Construction Kit* (Ambion Cat# 1620).

Briefly, oligonucleotides were dissolved in nuclease-free water and the concentration of the oligonucleotides was determined by reading the absorbance at 260 nm. The oligonucleotides were incubated with T7 promoter primer and DNA hybridization buffer at 70$^{o}$ C for 5 min, and then left in the room temperature for another 5min. 10X Klenow reaction buffer, 10X dNTP and exo-Klenow were added into the tube and the tube was incubated in 37° C for 30 min. After the siRNA templates were prepared, they were incubated with NTP mix, T7 reaction buffer and T7 enzyme mix at 37° C for 2 hrs for transcription reactions to synthesize the sense and antisense RNA strands of the siRNAs and then the sense and antisense transcription reactions were combined into a single tube and incubation continued at 37° C overnight. The dsRNAs were digested with DNase and RNase by incubating for 2 hrs at 37$^{o}$ C to remove the 5' overhanging leader sequences prior to transfection. The concentration of siRNA was determined by reading absorbance at 260 nm.

siRNAs were transfected into mammalian cells such as COS-7 cells by using siPORT *Amine transfection Agent* (Ambion Cat# 4502). Briefly, cells were plated at the concentration of 1 X 10$^{5}$/ml and the siPORT Amine transfection Agent was incubated 10 min at room temperature in OPTI-MEM from Gibco. The RNA was diluted in the medium to a final concentration of 30 nM and incubated for 10 min at room temperature. The RNA/transfection agent complexes were dispensed into the plates and prepared cells were transferred to these plates. The cells and the RNA/transfection agent complexes were gently mixed and incubated at 37$^{o}$ C until ready to assay. Western blotting described above was used to assay the efficiency of transfection.

***Electron microscopy***

Cells treated with different death inducers were pre-fixed in 2.5% glutaraldehyde in 0.2 M cacodylate buffer, pH 7.2, for 30 min, and then washed in cacodylate buffer twice. Cells were gently scraped off with a rubber policeman and centrifuged in Eppendorf tubes at 1,200g for 5min. The post-fixation was with 1% osmium tetroxide for 2 hrs at room temperature, after which the cells were washed with cacodylate buffer 3x and dehydrated through ascending ethanol concentrations (50%-100%) twice each for 5min. The samples were sent to Dr. Walter Malorni at Institute Superiore di Sanita, Italy for further processing. Samples were embedded in Epon 812 and thin sections were cut, after which the sections were stained with uranyl acetate and sodium citrate, and observed with Zeiss EM10 electron microscope at 8000X magnification.

## PART III. RESULTS AND DISCUSSION

### Chapter IV. Is Cdk5 activation dependent on caspase-3 activity during cell death induced by cyclophosphamide (CP)?

A. Neuronal cell death is induced by CP independent of caspase-3 activity.

B. Cdk5 activation is independent of caspase-3 activity during CP-induced neuronal death.

### Chapter V. Is Cdk5 activity required for induction of cell death?

A. Prevention of p25 generation by calpain inhibition does not affect induction of cell death.

B. Activation of Cdk5 during cell death does not require calpain activity.

C. Prevention of p25 generation does not alter the type of cell death.

D. Deregulation of Cdk5 activity by RNAi does not affect cell death induced by CPT.

### Chapter VI. Is Cdk5 activity dependent on cathepsin activity?

A. Determination of the level of cell death induced by different stimuli in cathepsin B, D, or L null and wild type cells.

B. Identification of the type of cell death induced by different stimuli in cathepsin B, D, or L null and wild type cells.

**C.** Examination of expression and activation of Cdk5 during cell death in cathepsin B, D, or L null and wild type cells.

## Chapter IV. Is Cdk5 activation dependent on caspase-3 activity during cell death induced by cyclophosphamide (CP)?

A. Neuronal cell death is induced by CP independent of caspase-3 activity.

B. Cdk5 activation is independent of caspase-3 activity during CP-induced neuronal cell death.

## 1. Abstract

Previous studies from our and other laboratories demonstrated a correlation between Cdk5 activity and cell death in many systems, including mouse brain, the interdigital area of mouse limb, rat lens, atrophic mouse ovarian follicles, neuronal injuries and neurodegenerative disorders (Ahuja et al., 1997; Zhu et al., 2002; Cheung et al., 2006; Lin et al., 2006). Although different insults result in the activation of different cell death signaling pathways, they often converge on the activation of caspases. Caspases are among the most important proteases in cell death. We have shown that activation of Cdk5 is independent of caspase-3 by using caspase-3 null fibroblast cell lines killed by different toxins (Lin et al., 2006). However, we have reported results only for cells that are not neurons and in culture.

Cdk5 is known as neuronal Cdc-2-like kinase (Nclk) since its sequence is very similar to that of Cdc-2 (Lew et al., 1992). Cdk5 is also known as an essential molecule in brain and its activity is important for neuronal development (Guo, 2006). The relationship between Cdk5 activity and caspase-3 activity during neuronal cell death in embryos remains unclear and was the aim of this study. We explored the association of Cdk5 activity and caspase-3 activation during cyclophosphamide (CP)-induced death of neurons in developing caspase-3 knockout transgenic mouse embryos. CP kills many cells in mouse embryos, and this cell death is apoptotic (Zhu et al., 2002). Thus this model is suitable for studying of the correlation between Cdk5 activity and caspase-3 activity during cell death.

To evaluate whether Cdk5 activation is dependent on caspase-3 activity during cell death in developing neurons, we examined the activation of Cdk5 and DNA fragmentation in different brain regions of both caspase-3 +/+ and caspase-3 -/- mouse embryos. The level of Cdk5 activation correlates with the level of neuronal cell death in both caspase-3 +/+ and caspase-3 -/- mouse embryos. However, we find that Cdk5 activation is independent of caspase-3 during the normal programmed cell death of neurons as well as in neuronal death induced by CP.

Taken together, in this section we demonstrate that Cdk5 activation was not regulated by caspase-3 during neuronal death, and that its activity may be upstream of caspase-3 activation.

## 2. Results

### A. Neuronal cell death induced by CP is independent of caspase-3 activation.

To establish a model in which we could examine the dependency on caspase-3 of the activation of Cdk5 during neuronal death, we used caspase-3 +/+ and caspase-3 -/- transgenic mouse embryos exposed to cyclophosphamide (CP). CP is a teratogen, the cytotoxicity of which is thought to mainly result from DNA double strand crosslinks (Ojwang et al., 1989) and DNA strand breaks (Verly, 1974; Colvin, 1993). CP is toxic to cultured neurons and to the developing CNS of rat (Rzeski et al., 2004). We have shown that CP induces apoptosis and increases the amount of cell death in an embryo many fold (Zhu et al., 2002). The question we asked here was whether CP could induce neuronal death in both caspase-3 +/+ and caspase-3 -/- mouse embryos and whether this death was indeed apoptotic.

Developing mouse embryos at ED 12.5 were exposed to CP in utero for 18 hrs. The embryos were removed and tails were taken to obtain DNA for genotyping. Caspase-3 +/+ and caspase-3 -/- mouse embryos were selected for sectioning according to genotyping. In order to detect neuronal death, future cerebral cortex (CC), midbrain (MB), and trigeminal ganglia (TG) were selected for staining. The layered structure of mature cerebral cortex is formed by migration of pyramidal cells. The migration, arrest, and ultimately positioning of cortical neurons require signaling activity from cyclin-dependent kinase 5 (Cdk5), and mice deficient in Cdk5, p35, or both p35 and p39 display cortical layer inversion, neuronal disorientation, and abnormal fiber infiltration (Hammond et al., 2004). Trigeminal ganglia are sensory ganglia comprised of different

cell types, such as satellite cells and Schwann cells. Caspase-3 activation has been shown during cell death in trigeminal gangliogenesis (Konishi et al., 2006). In developing mouse nervous system, massive cell death in the trigeminal ganglia is accompanied by the increased Cdk5 expression and induced Cdk5 activation (Zhang et al., 1997). Substantia nigra is a heterogeneous portion of the midbrain and a major element of the basal ganglia. Increased expression of Cdk5 and its activator, p35, is found in induced apoptotic neuronal death followed by intrastriatal injection of 6-hydroxydopamine and axotomy in living brain (Neystat et al., 2001).

Cell death was examined by TUNEL (Terminal deoxynucleotidyl transferase dUTP Nick Ending Labeling), which can show DNA fragmentation, one of the biochemical characteristics of apoptotic cell death (Saraste & Pulkki, 2000). TUNEL staining revealed a large number of cells in CC, MB and TG regions exhibiting fragmented DNA in caspase-3 +/+ control (Fig. 1A, B, & C) and CP-treated developing embryos (Fig. 1D, E, & F), and also in caspase-3 -/- controls not exposed to any toxins (Fig. 2A, B, & C) and CP-treated developing embryos (Fig. 2D, E, & F), which suggests that cells were undergoing apoptosis with or without caspase-3 activation during normal programmed neuronal death and CP-induced neuronal death.

We quantified the positive signals by counting 3-5 fields in different sections under the microscope at (400X) of untreated control and CP-treated caspase-3 +/+ and caspase3 -/- embryos. In all three brain regions (CC, MB, and TG) of the CP-treated caspase-3 +/+ and caspase-3 -/- embryos, there was significantly increased cell death when compared to the untreated caspase-3 +/+ (Fig. 3A) and caspase-3 -/- controls (Fig. 3B), indicating that in this system CP can induce apoptosis regardless of the availability

of caspase-3. However, the level DNA fragmentation was significantly higher in the CP-treated caspase-3 +/+ embryos than in the CP-treated caspase-3 -/- embryos (Fig. 3C), indicating that absence of caspase-3 partially inhibited DNA fragmentation. So, CP induces substantial cell death. The level of DNA fragmentation in CC is significantly higher than in TG and MB regions in caspase-3 +/+ embryos treated with CP and the level of DNA fragmentation is decreased most in the CC by caspase-3 knockout (Fig. 3D), suggesting that cell death in CC regions is more dependent on caspase-3 activity.

Functional caspase-3 is essential for the formation of apoptotic bodies, chromatin condensation, and DNA fragmentation in most types of cells. However, we have shown above DNA fragmentation in caspase-3 -/- embryos. In order to confirm that caspase-3 activity is abolished in caspase-3 -/- mouse embryos and that DNA fragmentation can occur without caspase-3 activation, we looked for activated caspase-3 protein by immunohistochemistry using anti-active caspase-3 antibody. Adjacent sections of developing mouse embryos used for TUNEL staining were stained by immunohistochemistry in both untreated control and CP-treated caspase-3 +/+ and caspase-3 -/- mouse embryos. Caspase-3 +/+ mouse embryos exhibited substantially activated caspase-3 in TG, MB, and CC in both un-treated control (Fig. 4A, B, & C) and CP-treated embryos (Fig. 4D, E, & F), but no active caspase-3 signal was found in these regions in either un-treated control (Fig. 5A, B, & C) or CP-treated caspase-3 -/- embryos (Fig. 5D, E, & F). In caspase-3 +/+ mouse embryos, the level of caspase-3 activation is significantly higher in CP-treated mouse embryos compared to the level of caspase-3 activation in untreated mouse embryos in three brain regions (Fig. 6A). Also the level of caspase-3 activation in CC is higher than MB and TG (Fig. 6B). These findings indicate

that caspase-3 is activated in the wild type mouse embryos during neuronal cell death induced by CP. Caspase-3 activation is induced by CP during neuronal cell death with caspase-3 activation elevated much more in the CC. Second, our caspase-3 -/- mice indeed lack activation of caspase-3.

These results indicate that DNA fragmentation is partially independent of caspase-3 activation. We conclude that caspase-3 is not necessary for DNA fragmentation.

**B. Cdk5 activation is independent of caspase-3 activity during CP-induced neuronal death.**

Having established that neuronal death is induced by CP in caspase-3 -/- mouse embryos, we asked whether Cdk5 is expressed and activated during this cell death. Cdk5 activity was examined in caspase-3 +/+ and caspase-3 -/- embryos after exposure to vehicle or CP by immunohistochemistry using anti-Cdk5 antibody. Similar sections as analyzed by TUNEL for DNA fragmentation were tested for activated Cdk5 by immunohistochemistry. Others have demonstrated that Cdk5 identified by immunohistochemistry is indicative of kinase activity (Gao et al., 1997; Zhang et al., 1997). We found cells positive for Cdk5 protein in the CC, MB, and TG of caspase-3 +/+ untreated control (Fig. 7A, B, & C) and CP-treated (Fig. 7D, E, & F) embryos and also in the same regions of caspase-3 -/- untreated control (Fig. 8A, B, & C) and CP-treated (Fig. 8D, E, & F) mouse embryos, indicating that Cdk5 is activated independent of caspase-3 activity.

A t-test was used to statistically analyze the level of Cdk5 activation, similar to the determination of the level of DNA fragmentation above. This was done by comparing the number of positive signals of Cdk5 activation in the three brain regions studied from 3-5 fields in different sections at high magnification (400X). We found that Cdk5 activity is significantly increased in both CP-treated caspase-3 +/+ mouse embryos (Fig. 9A) and CP-treated caspase-3 -/- mouse embryos (Fig. 9B) during neuronal cell death. Comparison of the level of Cdk5 activation in CP-treated caspase-3 +/+ and caspase-3 -/- embryos (Fig. 9C), demonstrated that caspase-3 at least partially contributed to activation of Cdk5. CP induced the highest level of Cdk5 activation in the CC of caspase-3 +/+ embryos and the absence of caspase-3 activity the level of Cdk5 activity was much reduced (Fig. 9D) compared in TG and MB. Taken together, our findings indicate that CP can induce Cdk5 activation during cell death whether or not caspase-3 is activated. Activation of Cdk5 is highest and most dependent on caspase-3 activity in the CC compared to TG and MB.

## 3. Discussion

In this study we compared the levels of neuronal death induced by CP in wild type and caspase-3 null mouse embryos. Our results indicate that CP can induce either caspase-3 dependent or caspase-3 independent cell death. DNA fragmentation was seen in both cases, indicating that neuronal cell death induced by CP is apoptotic. The level of DNA fragmentation was significantly decreased in caspase-3 -/- mouse embryos, suggesting that apoptosis was decreased by the absence of caspase-3. Nevertheless, since DNA fragmentation is a marker for apoptosis, different pathways and types of cell death not detectable by TUNEL may operate in caspase-3 -/- mice, and we have not ruled out the possibility that the level of non-apoptotic cell death may not decrease in null mice. However, in our previous study when we analyzed by trypan blue exclusion death caused by CHX and CPT in caspase-3 -/- cells, total cell death was lower in caspase-3 -/- cells (Lin et al., 2006). This finding is consistent with the decreased level of DNA fragmentation in caspase-3 -/- mouse embryos exposed to CP, indicating that the level of cell death is less in the absence of caspase-3 activity. We also find that DNA fragmentation can occur without caspase-3 activation, suggesting that cell death induced by CP is apoptotic. Apoptotic cell death can be independent of caspase-3. Caspase activity may not be required for apoptotic cell death as was previously shown from the study of caspase knockout animals (Belmokhtart et al., 2001; Subramaniam & Unsicker 2006; Kumar 2007), and most of the cells derived from caspase-3 mutant mice eventually die by apoptosis (Kuida et al., 1996). Other proteases may play roles in DNA fragmentation during cell death. The compensatory activation of other effector caspases,

such as caspase-6 and caspase-7, was reported for liver caspase-3 deficient cells treated with anti-Fas antibody and UV irradiation (Zheng et al., 2000). In our experimental systems, the reduced level of cell death and DNA fragmentation induced by CP in embryos deficient in caspase-3 suggests that caspase-3 cannot be completely compensated by other enzymes during cell death. During neuronal death, caspase-3 activity is different in different regions of the brain and the cells in different regions are differentially sensitive to CP and to the lack of caspase-3 activity, indicating that cells in different regions of the brain may have quantitatively or qualitatively different regulatory signaling pathways. We have as yet no explanation for these differences.

Since Cdk5 has been shown to play an important role in the migration and positioning of cortical neurons during neuronal development (Hammond et al., 2004; Ohshima et al., 2007), in this study we also examined the Cdk5 activity in these brain regions to answer whether Cdk5 is related to these differences and to caspase-3 activity during cell death. We found that these regional differences apply also to Cdk5. We show highest Cdk5 activity in the CC during normal development, suggesting that Cdk5 plays an important role in cortical development. The level of DNA fragmentation and Cdk5 activation was lowered most in the CC by the loss of caspase-3 activation, indicating that caspase-3 activation is more important in DNA fragmentation and Cdk5 activation for the cells in this region and that CC is more sensitive to CP during development.

Cdk5 was activated in both caspase-3 dependent and caspase-3 independent cell death and the level of Cdk5 activation was consistent with the level of DNA fragmentation, indicating that Cdk5 is activated when cells die and that the activation of Cdk5 does not require caspase-3 activation. These data are consistent with the results we

obtained in other *in vitro* systems, that Cdk5 activation correlates with cell death in general aspects and is not dependent on caspase-3 activation. The independence of Cdk5 activation from caspase-3 during induced cell death may indicate that Cdk5 activation is upstream of caspase-3 activation in the cell death signaling transductions; that Cdk5 is involved in cell death signaling pathways paralleling to the caspase cascade; or that Cdk5 is activated because cells die but plays no functional role in the death.

However, the relationship between Cdk5 activation and caspase-3 activation is still unclear and conflicting results have been reported. Sandal et al, 2002 showed that in rat leukemia cells Cdk5 activation is located upstream of caspase-3 activation and that Cdk5 activity is necessary for the activation of caspase-3 in cAMP-induced apoptosis. In contrast, O'Hare et al., 2005 indicated that in primary neurons the production of p25, the activator of Cdk5 during cell death, is regulated downstream of caspase-3 activation because active caspase-3 degrades the endogenous calpain inhibitor calpastatin. To further evaluate whether Cdk5 activation is upstream of caspase-3 and affects caspase-3 activation; whether Cdk5 activation is irrelevant to caspase-3 activation; or whether Cdk5 activation is a result of cell death, in the next section we will study the inhibition of Cdk5 activity to detect caspase-3 activation during cell death. These findings will shed more light on the relationship between Cdk5 activation and caspase-3 activation during cell death, as well as the relationship between Cdk5 activation and cell death.

## Chapter V. Is Cdk5 activity required for induction of cell death?

A. Preventing p25 generation by inhibition of calpain does not affect induction of cell death.

B. Activation of Cdk5 during cell death does not require calpain activity.

C. Preventing p25 generation does not alter the type of cell death.

D. Deregulation of Cdk5 activity by RNAi does not affect cell death induced by CPT.

## 1. Abstract

Cyclin dependent kinase 5 (Cdk5) is a member of the cyclin dependent kinase (Cdk) family. However, unlike other Cdks, Cdk5 is involved not in the regulation of cell cycle, but in both cell differentiation and cell death (Guo, 2006; see introduction). The increase of Cdk5 expression and activation accompanies cell death in different models, including the interdigital areas of retinoic acid-treated developing mouse limb (Ahuja et al., 1997), in developing mouse embryos treated with cyclophosphamide (Zhu et al., 2002), as well as in all the other cell deaths seen during normal development (Lin et al., 2006). Cdk5 is activated not by cyclins, but by its own activators, such as p35 and p39 during neuronal development and their truncated fragments, p25 and p29, during the deaths of neurons and other cells. The cleavage of p35 and p39 into p25 and p29 requires the activation of calpain, which results in prolonged activation and neurotoxic Cdk5 activity (Ko et al., 2001; Patzke & Tsai, 2002; Lee et al., 2000; Fu et al., 2006). Our laboratory has shown that Cdk5/p25 activation correlates with cell death in toxin-exposed COS-7 cells (Zhu et al., 2002) and in different mouse embryonic fibroblast cell lines, including p53, Apaf-1, caspase-9 or caspase-3 deficient cells. We have shown that Cdk5 activation is independent of p53, Apaf-1, caspase-9 and caspase-3 (Lin et al., 2006).

From these studies, one can postulate that Cdk5 activation is an important aspect of many forms of cell death and that blocking the activators of Cdk5, such as by inhibition of calpain activity should inhibit the activation of Cdk5 and, if Cdk5 functionally contributes to the death, prevent cell death. To determine the consequence of inhibition of Cdk5 activity during cell death, we examined cell death following deregulation of Cdk5 activity. First, the activity of Cdk5 was reduced by inhibiting the

production of p25 through inhibition of calpain activity. We found that Cdk5 was activated even in the absence of p25 in the early stage apoptotic deaths (8 hrs exposure to CPT). However, activated Cdk5 disappeared in the late stage (18 hrs) exposure to CPT, by which time we found the conversion of apoptosis to other types of cell death. Then, to confirm that cell death is not affected by the inhibition of Cdk5 activation, we specifically inhibited Cdk5 activity by using RNA interference (RNAi) to block expression of Cdk5 message. We demonstrated that the level of cell death and the type of cell death were not altered by inhibition of Cdk5 expression.

We conclude that Cdk5 activity is not required for the induction of cell death and that Cdk5 can be activated by activators other than p25. In addition, cells can undergo apoptosis without Cdk5 activation.

## 2. Results

### A. Preventing p25 generation by inhibition of calpain does not affect induction of cell death

Cdk5 activity during cell death is normally regulated by the production of its activator, p25 and p29, truncated from p35 and p39 by calpain (Zhu et al., 2002; Tsai et al., 2004). We showed that in mouse embryonic fibroblast C8 cell lines inhibiting calpain activity by calpain inhibitor (PD150606, PD) inhibits the generation of p25 during cell death induced by CPT (Lin et al. 2006), which makes it possible to examine the effect of the prevention of p25 generation on cell death using this model.

After C8 cells reached 80% confluence, cells were switched to media with low serum (1% FBS) and exposed to CPT (50 μM) in the absence or presence of PD (15 μM). After 8 and 18 hrs exposure, cells were collected and cell death was determined by trypan blue assay. As shown in Fig. 10, the untreated control and PD-treated cells showed 7.6% and 7.7% cell death after 8 hrs treatment respectively, and 15.0% and 15.7% cell death after 18 hrs treatment respectively, indicating that C8 cells are sensitive to low serum concentration (1% FBS during treatment) and that PD does not induce cell death compared to untreated control cells. The total cell death in C8 cells treated with CPT (50 μM) was 43.0% after 8 hrs exposure and 90.2% after 18 hrs exposure. The amount of cell death induced by CPT (50 μM) in the cells in the presence of PD (15 μM) after 8 hrs was 44.4%, and 90.6% after 18 hrs exposure (Fig. 10). Comparing the level of cell death induced by CPT in the presence or absence of PD, inhibiting the generation of p25 by PD did not change the amount of cell death in C8 cells. Preventing the production of p25

does not affect the induction of cell death by CPT. Therefore, we asked whether Cdk5 activation is indeed blocked by inhibiting calpain.

**B. Activation of Cdk5 during cell death does not require calpain activity.**

To examine whether the activation of Cdk5 requires calpain activity and the generation of p25, we determined Cdk5 activation by kinase assay during cell death.

From cells treated with CPT (50μM) in the presence or absence of PD (15 μM) for 18 hrs, protein samples were extracted and Cdk5 protein was immunoprecipitated from the cell lysates and incubated with the substrate of Cdk5, histone H1. The protein samples were run on 12.5% SDS-PAGE and analyzed by western blot using anti-phospho-histone H1 antibody to determine whether histone H1 was phosphorylated by active Cdk5. For 8 hrs treatment with CPT in the presence or absence of PD, Cdk5 kinase activation was detected by the appearance of the phosphorylated histone H1 at 31 kDa (Fig. 11A). The level of Cdk5 activation in CPT + PD treated cells was similar to that in the cells treated only with CPT (Fig. 11A), indicating that the inhibition of calpain did not affect the activation of Cdk5 during cell death at 8 hrs treatment. However, in the samples from cells exposed to CPT in the presence or absence of PD for 18 hrs, the activation of Cdk5 was found in the sample treated with CPT alone, but not in the sample treated with both CPT and PD (Fig. 11B), indicating that activated Cdk5 is lost after 18 hrs exposure to CPT when calpain activity is inhibited. Therefore, we asked whether the expression of Cdk5 is inhibited in this situation. Equal amounts of cell lysates from cells treated with CPT in the presence or absence of PD for 18 hrs were run on a 12.5% SDS-PAGE gel. The expression of Cdk5, demonstrated by western blot using anti-Cdk5

antibody, showed a similar level of Cdk5 protein expression in both untreated control and treated cells (Fig. 11C), suggesting that Cdk5 expression is not prevented by calpain inhibitors during cell death.

Thus, Cdk5 activation is not affected by the inhibition of p25 generation at early stage (8 hrs exposure), indicating the presumptive presence of activators of Cdk5 other than p25 during cell death. Cdk5 activation is lost after 18 hrs exposure of CPT and PD, indicating that the half-life of this activator, the production of which does not require calpain activation during cell death, is shorter than that of p25, and that cell death occurs independent of Cdk5 activation. However, we do not know whether cells take the same path or different paths to die. The same amount of cell death is induced by CPT when Cdk5 activation is lost by 18 hrs. To answer the question of the type of death, we investigated the type of cell death by different means as is discussed below.

**C. Preventing the generation of p25 does not alter the type of cell death.**

There are several hallmarks characterizing cell death, among which nuclear condensation and nuclear fragmentation are often used. The changes of the nucleus during cell death induced by CPT were detected by Hoechst staining. Nuclear condensation and nuclear fragmentation were shown in C8 cells treated with CPT for 18 hrs in both the presence (Fig. 12C) and absence (Fig. 12D) of PD by comparing them to the normal cells in the untreated control cells (Fig. 12A & B). Inhibition of p25 production by calpain inhibitor does not affect the changes of the nucleus during cell death induced by CPT. Since the appearance of some of the classical apoptotic morphology is attributed to caspase-3 activation during cell death (Leist & Jaattela,

2001), we further evaluated caspase-3 activation during cell death induced by CPT in cells lacking calpain activity. From C8 cells incubated with CPT (50 μM) for 8 hrs and 18 hrs in the presence or absence of PD, protein samples were isolated from cells with RIPA buffer and equal amounts of samples were run on 15% SDS-PAGE. Caspase-3 activation was detected by western blot using anti-active caspase-3 antibody. The band of the active form of caspase-3 at 17 kDa was seen in cells exposed to CPT with or without calpain inhibitor (PD) for 8 hrs and 18 hrs (Fig. 13A). The level of caspase-3 activity of the cells with and without PD was similar, indicating that the inhibition of calpain activity has no effect on the activation of caspase-3. However, the level of active caspase-3 at 18 hrs exposure to CPT was lower than at 8 hrs exposure (Fig. 13A), presumably owing to the degradation or loss of caspase-3 at late stages of cell death.

To further confirm that cell death induced by CPT is not altered in cells with deregulated Cdk5 activity is not altered and that apoptotic cell death was induced by CPT, we observed the detailed morphological changes in dying cells by electron microscopy. Since there are specific morphological changes that occur during different types of cell death, EM analysis can provide direct evidence of the mode of cell death. Electron microscopic analysis was also performed in C8 cells treated with CPT for 8 hrs and 18 hrs in the presence or absence of PD. As compared to the untreated control normal cells (Fig. 14A), CPT-treated cells in both presence and absence of PD at 8 hrs treatment showed apoptotic features in the dying cells, displaying condensed and fragmented marginated chromatin, cell rounding and cell shrinkage, and vacuolization of the cytoplasm (Fig. 14C & E). After 18 hrs exposure to CPT, there were signs of secondary necrosis, such as the disintegration of the outer plasma membrane and diffuse appearance

of the chromatin, in CPT-treated cells in the presence and absence of PD. These findings suggest that the advanced stage of CPT-induced cell death is necrosis (Fig. 14D & F). Data from EM analysis indicate that the inhibition of calpain activity did not alter the type of cell death induced by CPT.

**D. Deregulation of Cdk5 activity by RNAi does not affect cell death induced by CPT.**

To more specifically examine the role of Cdk5 in cell death we directly reduced its amount though the use of RNA interference. Five dsRNAs were synthesized from five different oligonucleotides designed from target Cdk5 sequences according to Cdk5 DNA sequence as described in Materials and Methods:

Cdk5-1 target sequence: AAGAUUGGGGAAGGCACCUAU;

Cdk5-2 target sequence: AAGGCACCUAUGGAACUGUGU;

Cdk5-3 target sequence: AAACCGGGAAACUCAUGAGAU;

Cdk5-4 target sequence AAACUCAUGAGAUUGUGGCUC;

Cdk5-5 target sequence AAGAAAUAUUUCGACAGCUGC.

These dsRNA were transfected into COS-7 cells. After transfection, the protein expression of Cdk5 was measured by western blot using anti-Cdk5 antibody. We found that one of the dsRNAs (Cdk5-1, Fig. 15Aa) substantially lowered the expression of Cdk5 proteins, as indicated by the density of the bands and measured by densitometry (Fig. 15Ab). Cdk5 expression in Cdk5-1 transfected cells decreased to 16.2% of that in the non-transfected control. dsRNA of Cdk5-3 inhibited Cdk5 expression to 40% but Cdk5-2 dsRNA does not block expression of Cdk5 (Fig. 15Ab).

Three dsRNAs (Cdk5-1, Cdk5-2, and Cdk5-3) were used for the experiments. Since Cdk5-1 and Cdk5-3 can inhibit Cdk5 expression, but Cdk5-2 has no effect on the expression of Cdk5, Cdk5-2 was used as control to examine whether the transfection affects cell death. The percentage of cell death was determined by trypan blue exclusion in the COS-7 cells transfected with dsRNAs (Cdk5-1, Cdk5-2, and Cdk5-3). A cell death inducer, CPT (25 μM), was used for 24 hrs and 48 hrs, since we found that less cell death can be induced at 24 hrs and more cell death can be induced at 48 hrs. As shown in Fig. 15B, 8.2% of non-transfected COS-7 cells died by 24 hrs and 15.2% by 48 hrs without CPT-exposure presumably because of low FBS during treatment. In non-transfected COS-7 cells, CPT can induce 25.4% cell death by 24 hrs after CPT exposure and 76.4% cell death by 48 hrs. After 24 hrs and 48 hrs exposure to CPT, there was showed 58.1% and 88.9% cell death, respectively, in cells transfected with dsRNA Cdk5-1; 55.3% of cell death by 24-hrs exposure and 92.4% of cell death by 48-hrs exposure of CPT in cells transfected with dsRNA Cdk5-2; 56.3% of cell death by 24 hrs exposure and 90.1% of cell death by 48 hrs exposure of CPT in cells transfected with dsRNA Cdk5-3. Control cells not exposed to CPT but transfected with Cdk5-1, Cdk5-2 and Cdk5-3 displayed 26.0%, 25.7% and 22.9% cell death by 24 hrs respectively, and 32.2%, 31.7% and 28.8% cell death by 48 hrs respectively. By comparing the level of cell death between transfected and non-transfected CPT-treated and untreated control cells, approximately 15% more cell death occurred in transfected cells than non-transfected cells irrespective of CPT treatment, a result that indicated that the transfection can increase the level of cell death. However, inhibition of Cdk5 expression by dsRNA did not affect the level of cell death induced by CPT. We conclude that down-regulation of Cdk5 expression by dsRNA

directly does not alter the level of cell death induced by CPT. Again whether this cell death represents the same type of cell death as seen with activated Cdk5 needs to be investigated.

To test the hypothesis that deregulation of Cdk5 activity does not change the type of cell death we used different markers of cell death, including nuclear fragmentation by Hoechst staining, activation of caspase-3 by western blot and morphological changes by EM analysis. First, cells transfected with Cdk5-1 and Cdk5-2 were exposed to CPT (25 μM) for 48 hrs and fixed for Hoechst staining. As shown in Fig. 16, nuclear condensation and nuclear fragmentation were detected in both the COS-7 cells transfected with Cdk5-1and Cdk5-2 and non-transfected cells during cell death induced by CPT, indicating that the inhibition of Cdk5 expression did not affect the changes of nucleus during cell death induced by CPT, and that cell death induced by CPT may be apoptotic.

We determined caspase-3 activation by western blot using anti-active caspase-3 antibody during cell death induced by CPT. Caspase-3 activation is essential for some of the classical changes of apoptosis (Leist & Jaattela, 2001). COS-7 cells were treated with CPT (25μM) in the absence or presence of dsRNAs (Cdk5-1, Cdk5-2, and Cdk5-3) of Cdk5 for 48 hrs and then protein samples from COS-7 cells were separated on 15% SDS-PAGE and analyzed by western blot using anti-active caspase-3 antibody. The appearance of a band at 17 kDa indicated the production of active form of caspase-3 from the cleavage of procaspase-3, the size of which is 35kDa in CPT-treated cell samples with or without dsRNA (Cdk5-1, Cdk5-2, and Cdk5-3) transfection (Fig. 17). This finding indicates that CPT induces caspase-3 activation during cell death in cells irrespective of 84% downregulation of Cdk5 expression by RNAi

Finally, the detailed morphological changes were observed by electron microscopy. As mentioned before, the apoptotic cell displays cell rounding, chromatin condensation and fragmentation as well as chromatin margination. In both Cdk5-1- and Cdk5-2-transfected, untreated control cells, EM analysis showed elongated cells with normal morphology (Fig. 18A & B), indicating that transfection of Cdk5 did not affect the morphology of cells under normal conditions without stress. After cells were exposed to CPT for 48 hrs, the dying cells had apoptotic morphology including condensed and fragmented chromatin and rounding and shrinkage similar to that of nontransfected cells exposed to CPT (Fig. 18C, D). These findings indicate that the downregulation of Cdk5 did not affect the type of cell death and that cells can still die through apoptosis.

We can conclude that cells can still die and that de-regulation of Cdk5 activity by calpain inhibition and down-regulation of Cdk5 by dsRNA does not alter the type of cell death. Therefore, Cdk5 is not involved in the regulation of cell death.

### 3. Discussion

The amount of cell death in cells treated with calpain inhibitor, PD, is not reduced in either untreated control or CPT-treated cells. The finding that inhibition of p25 and p29 generation by calpain inhibitor (Fu et al., 2006; Lin et al., 2006) does not prevent the generation of the hallmarks of apoptotic cell death, caspase-3 activation and nuclear condensation and fragmentation, during cell death induced by CPT, suggests that the inhibition of Cdk5/p25 and Cdk5/p29 activity by calpain inhibitor does not affect the type of cell death and that cells may die through the same signaling pathways. Since Cdk5/p25 activity was blocked by calpain inhibitor (Lin et al., 2006), the unchanged level of cell death and nuclear fragmentation may result from the existence of other Cdk5 activators during cell death or the existence of a Cdk5-independent pathway. We found activation of Cdk5 when Cdk5/p25 and Cdk5/p29 activity is inhibited and further confirmed the hypothesis that Cdk5 can be activated by activators other than p25 and p29 during cell death. Recently, RINGO, a *Xenopus* protein, was shown to activate Cdk5 in *Xenopus* oocytes (Dinarina et al., 2005; Nebreda, 2006). It has been mentioned that other proteins, such as TNF-α and caspase-3, can increase Cdk5 activity (Orenllana et al., 2007; O-Hare et al., 2005). p35 which is involved in cell differentiation, is less stable than p25 and is degraded faster than p25. Cdk5/p35 activity is also related to cell death. For example, enhancement of Cdk5 and p35 immunoreactivity is observed in the apoptotic cell body following ischemia (Hayashi et al., 1999). Elevated Cdk5 and p35 activity are detected in dying oocytes (Zhang et al., 1997). It appears that the activator that compensates the role of p25 during cell death induced by CPT in C8 cells may be p35.

At late stages of cell death (18 hrs exposure to CPT), activated Cdk5 is lost when cells were treated with calpain inhibitor, but the level of cell death is not decreased. Furthermore, when Cdk5 expression was inhibited by dsRNA the amount of induced cell death by CPT at 48 hrs exposure is 61.1% in non-transfected cells and the amount of induced cell death by CPT is 56.7% in Cdk5-1 dsRNA Cdk5transfected cells, which is consistent with the results from the study with calpain inhibition. These results suggest that the level of cell death induced by CPT is not affected by the inhibition of Cdk5 expression and that cells can die independent of Cdk5. Neurons from Cdk5 knockout mice can undergo apoptosis indicating the existence of a Cdk5-independent cell death pathway (Li et al., 2002).

The results from EM analysis, detection of caspase-3 activation and Hoechst staining indicate that the inhibition of Cdk5 expression does not affect nuclear fragmentation during cell death or the type of cell death; and dead cells show apoptotic characteristics. These findings indicate that cells die through the same signaling pathways with or without Cdk5 activity. Taken together, although Cdk5 is activated during cell death, cells can die without Cdk5 activity and the type of cell death is not altered by inhibition of Cdk5 activity, which indicates that Cdk5 activation may not be involved in the regulation of cell death pathways but rather that the activation is a result of cell death.

Our studies also reveal that the level of caspase-3 activation is very similar when cells with normal Cdk5 expression are compared to those with reduced Cdk5 expression, suggesting that the activation of caspase-3 is independent of Cdk5 and that Cdk5 activity is not upstream of caspase-3 in the cell death signaling pathways. Cdk5 activity does not regulate the cell death related proteins in the cell death signaling pathways, such as

caspase-3, that generate the morphological characteristics of different types of cell death. In Chapter VI, we showed that Cdk5 activation is not downstream of caspase-3 in the cell death signaling pathway, suggesting that Cdk5 may be involved in a caspase-3 independent pathway or that its activity is not involved in the regulation of cell death, but rather that Cdk5 is regulated by cell death. To further evaluate the regulation of Cdk5 activity in cell death pathways, we will observe whether lysosomal proteases, such as cathepsins, are involved in the regulation of Cdk5 activity during cell death.

**Chapter VI. Is Cdk5 activity dependent on cathepsin activity?**

A. Determination of the level of cell death induced by different stimuli in cathepsin B, D, or L null and wild type cells.

B. Identification of the type of cell death induced by different stimuli in cathepsin B, D, or L null and wild type cells.

C. Examination of expression and activation of Cdk5 during cell death in cathepsin B, D, or L null and wild type cells.

## 1. Abstract

In the previous chapters, we have shown that Cdk5 is activated during cell death induced by CP in caspase-3 -/- mouse embryos and the downregulation of Cdk5 activity did not affect the activation of caspsae-3. In addition, studies from our lab have suggested that Cdk5 activation does not require caspase-9, Apaf-1 or p53 during cell death induced by different stimuli in different cell lines (Lin et al., 2006). We have also shown that calpain, a protease involved in the cleavage of p35 and p39 to p25 and p29 for the activation of Cdk5 during cell death, is not required for activation of Cdk5 during cell death induced by CPT (see Chapter V). However, other proteases whose activities have been associated with different paths leading to cell death, such as cathepsins (which are lysosomal proteases) may be related to the regulation of the activity of Cdk5 during cell death.

Lysosomes and lysosomal enzymes are involved in cell death. Lysosomal leakage is not only a downstream event of cell death, but also can initiate cell death (Kroemer & Jaattela 2005). “Lysosomal pathways of cell death” has become a new terminology accepted by more and more investigators. Cathepsins are the major class of lysosomal proteases; they have been linked with cell death. Especially, the most abundant lysosomal proteases including the cysteinyl proteases cathepsin B and L and the aspartyl protease cathepsin D can trigger a complete spectrum of cell death from necrosis to classic apoptosis (Frehrenbacher et al., 2004). The release of cathepsin from lysosomes results in the cleavage of their substrates resulting in the activation of the enzymes such as caspase-11 (Schotte et al., 1998) and granzyme A and B (Tardy et al., 2006). Cathepsin B, D, and

L play a role in programmed cell death during embryogenesis. Activation and release of cathepsin D is correlated with cell death in developing chick and duck embryos (Zuzarte-Luis et al., 2007). Knockout cathepsin B -/- and cathepsin L -/- mice displayed dramatic neurodegeneration (Chwieralski et al., 2006). There are a few reports on the relationship between Cdk5 and cathepsins.

To examine the relationship between Cdk5 and cathepsins, we used cathepsin B -/-, D -/- and L -/- mouse fibroblasts. We explored how the activity of Cdk5 during cell death induced by CHX and CPT depended on cathepsin B, D, and L. We found that lack of cathepsin B, D, or L delays cell death, but when cell death finally occurred Cdk5 was activated. Furthermore, we saw atypical morphological changes of apoptosis during cell death in cells lacking cathepsin B, D, or L.

## 2. Results

### A. Determination of the level of cell death induced by different stimuli in cathepsin B, D, or L null and wild type cells.

In order to examine if Cdk5 activity is regulated by cathepsins during cell death, we first established the model system, in which cell death was induced by cycloheximide (CHX) or camptothecin (CPT) in mouse embryonic fibroblast cells. The cells were taken from transgenic mice: cathepsin B, D, or L single knockouts, and the cells were immortalized with SV40 (Gift from Dr. Marianne Boes, Harvard Medical School). CHX is an inhibitor of protein biosynthesis (Gong et al. 1993), and CPT inhibits DNA replication by inhibiting DNA topoisomerase I (Morris et al. 2001).

Cathepsin B, D, L -/- and wild type cells were exposed to CHX (100 μg/ml) or CPT (50 μM) for 8, 24, 48, 72, 96, and 120 hrs. The amount of cell death was determined by using trypan blue exclusion assay (Material and Methods). The level of cell death in untreated control cells due to low serum concentration (1% FBS during treatment) was counted as the basal amount of cell death for each cell type and was subtracted from the total cell death to determine the cell death induced by CHX or CPT. As shown in Fig. 19, no dramatic amount of cell death was induced by CHX or CPT in any type of cells after 8-hrs treatment. From 24 hrs to 72 hrs exposure with CHX or CPT, the amount of induced cell death increased from 45.0% to 87.6% (Fig. 19A) and from 19.3% to 86.5% (Fig. 19B), respectively in wild type cells. However, in cathepsin B, D, and L -/- cells the amount of induced cell death did not increase as much as wild type cells. From 24 hrs to 72 hrs, the level of induced cell death in cathepsin B, D, and L -/- cells with CHX

treatment increased from 2.7% to 27.9%, from 7.5% to 28.9%, and from 3.7% to 34.1%, respectively (Fig. 19A). CPT killed 3.6% of cathepsin B-/- cells by 24 hrs and 34.7% by 72 hrs; 6.5% of cathepsin D -/- cells by 24 hrs and 32.9% by 72 hrs; and 2.6% of cathepsin L -/- cells by 24 hrs to 41.4% by 72 hrs (Fig.19B). By comparing the amount of induced cell death in all these cells treated with CHX or CPT, cells without cathepsin B, D, or L proved less sensitive to CHX or CPT. In order to evaluate whether cell death was inhibited or delayed in cathepsin B, D, or L -/- cells, the cathepsin B, D, and L -/- cells were incubated with CHX or CPT for 96 hrs and 120 hrs. After 120 hrs exposure, the amount of cell death induced by CHX can reach 70.9% in cathepsin B -/- cells; 63.6% in cathepsin D -/- cells; and 61.9% in cathepsin L -/- cells (Fig. 19A), and CPT can kill 76.8% of cathepsin B -/- cells; 71.6% of cathepsin D -/- cells; and 61.6% of cathepsin L -/- cells by 120-hrs exposure (Fig. 19B). These figures can be compared to the amount of cell death in wild type cells, 87.6% or 86.5% of induced cell death was obtained after 72 hrs exposure to CHX or CPT, respectively.

CHX or CPT induced different but substantial amounts of cell death in wild type cells and cathepsin knockout cells. Thus cells can die without cathepsin B, D, or L, though death is delayed.

**B. Identification of the type of cell death induced by different stimuli in cathepsin B, D, or L null and wild type cells.**

Having established that we could induce substantial cell death in cathepsin B, D, or L -/- cells, we asked if the death was apoptotic. For this analysis, we used several markers of apoptosis, such as examination of DNA fragmentation by TUNEL, nuclear

fragmentation by Hoechst staining, activation of effector caspases by western blot analysis, and cell morphology by electron microscopic analysis.

Cells were grown on coverslips to 80% confluence and exposed to CHX (100 μg/ml) or CPT (50μM) for 24 hrs and 72 hrs, and fixed with 3% paraformaldehyde as described in Materials and Methods. TUNEL assay was performed on the cells for the detection of fragmented DNA. We found positive signals as dark brown staining in both of the CHX or CPT-treated cells of wild type (Fig. 20) and cathepsin B -/- (Fig. 21), D -/- (Fig. 22) or L -/- (Fig. 23), indicating that DNA fragmentation was induced by CHX or CPT and did not require the activity of cathepsin B, D, or L during cell death.

In addition to DNA fragmentation, nuclear fragmentation was also evaluated by Hoechst staining (Materials and Methods) in wild type cells and cathepsin B, D, or L -/- cells exposed to CHX (100 μg/ml) or CPT (50 μM) for 8, 24, and 72 hrs and the number of fragmented nuclei per field was counted in 3-5 fields in different experimental preparation at 400X magnification. As shown in Fig. 24A, in WT cells, CPT induced nuclear fragmentation 5.8% by 8 h, 13.0% by 24 hrs, and 75.0% by 72 hrs. In cathepsin B, D, or L -/- cells, nuclear fragmentation induced by CPT from 8 hrs to 72 hrs exposure is from 0.4% to 4.2%, from 0.1% to 3.9%, and from 1.2% to 2.7% respectively. After exposure to CHX from 8 hrs to 72 hrs, the amount of nuclear fragmentation ranges from 4.1% to 89.0% in WT cells, to 0.2% to 6.3% in cathepsin B null cells; 0.6% to 4.8% in cathepsin D null cells; and from 0.1% to 3.5% in cathepsin L null cells, respectively (Fig. 24B). Our findings indicate that nuclear fragmentation is inhibited in cells lacking cathepsin B, D, or L even though cell death can be detected by trypan blue and DNA

fragmentation takes place in these cells, suggesting that cathepsins are involved in the regulation of nuclear fragmentation.

Activation of caspases is regarded to a hallmark for apoptotic cell death (Lakhani et al., 2006). The activation of effector caspases such as caspase-3 and caspase-7 is downstream of the cell death signaling pathways. Caspase-7 is very similar to caspase-3. These two caspases have similar substrate specificities (Fuentes-Prior & Salvesen, 2004), and their activation results in the irreversible cell death by cleavage of specific death substrates including poly(ADP)-ribose polymerase (PARP) as discussed in introduction (Kumar 2007). We examined the activation of caspase-3 and caspase-7 during cell death induced by CHX or CPT in WT and cathepsin B, D, or L null cells. After exposure of cells to CHX (100 μg/ml) or CPT (50 μM) for 24 and 72 hrs, cell lysates were prepared from both wild type and cathepsin null cells for western blot analysis. First, activation of caspase-3 was determined by using anti-active caspase-3 antibody. We detected no band at 17kDa in wild type cells (Fig. 25A), cathepsin B -/- cells (Fig. 25B), cathepsin D-/- cells (Fig. 25C), or cathepsin L -/- cells (Fig. 25D). The lack of caspase-3 activation is not due to our experimental procedure or the antibodies as we can detect positive signal in control samples of C8 cells induced to die by CPT, in which we have shown caspase-3 activation during cell death (Lin et al., 2006). To answer whether the lack of cathepsin B, D, or L interferes with the activation of caspase-3, we examined our cells are deficient in caspase-3 gene due to the preparation of the primary cells. We searched for the caspase-3 gene using PCR (same protocol and primers used for genotyping mouse embryos, see Chapter IV, Materials and Methods). We found that caspase-3 gene was absent in wild

type cells, and cathepsin B, D, and L -/- cells (Fig. 25E), which indicates that cathepsin null cells are also caspase-3 null cells.

As mentioned above, caspase-7 is also an effector caspase, and caspase-7 may compensate the function of caspase-3 (Degterev et al., 2003). Therefore, we examined the activation of caspase-7 in these cells during cell death. Equal amounts of protein from both wild type and cathepsin B, D, or L null cells after 72-hrs exposure of CHX or CPT were separated by 15% SDS-PAGE. The caspase-7 activation was examined by western blot using anti-active caspase-7 antibody and the appearance of a band at 19 kDa in both WT and cathepsin null cells indicated the activation of caspase-7 (Fig. 26A & D). The basal level of caspase-7 activation in untreated control cells (Fig. 26A & D) corresponded with the cell death due to low serum incubation for 72-hrs exposure. The activation of caspase-7 is further confirmed by examining the cleavage of PARP, a substrate for both caspase-3 and caspase-7 (Fuentes-Prior & Salvesen, 2004). Equal amounts of protein from wild type and cathepsin B, D or L -/- cells exposed to CHX or CPT for 72 hrs were run on a 8% SDS-PAGE and analyzed by western blot using anti-PARP antibody. The appearance of a band at 85kDa indicated that PARP was cleaved when cells were induced to die (Fig. 26B & E). These findings suggest that cell death induced by CHX or CPT in cathepsin knockout and wild type cells activates the caspase-7 or other caspases that are able to cleave PARP, although caspase-3 is absent from cathepsin knockout cells.

Transmission electron microscopy (TEM) was used to further characterize the type of cell death. EM analysis was performed in wild type and cathepsin knockout cells before and after treatment of CHX or CPT for 72 hrs. As shown in Fig. 27, we found loss of cell asymmetry and induction of cell rounding after treatments with both CHX or CPT

in wild type cells undergoing the typical changes occurring in apoptosis, including chromatin condensation, marginalization and clumping, accompanied with fragmentation of the nuclei and cytoplasmic changes such as condensation and general degeneration including cell shrinkage, finally leading to secondary necrosis (Fig. 27B & C). However, in the cells lacking cathepsin B (Fig. 28), cathepsin D (Fig. 29), and cathepsin L (Fig. 30), we observed different morphological changes, such as chromatin redistribution without any signs of either nuclear condensation or nuclear fragmentation (Fig. 28C; Fig. 29C & Fig. 30C), and the presence of vacuoles of different sizes in the cytoplasm, containing cell organelles in some of vacuoles (Fig 28B & Fig. 30C). These findings indicated that lack of cathepsin B, D, or L affected the changes of morphology during cell death and dying cells do not manifest the typical apoptotic characteristics seen in wild type cells.

In addition to the increase of vacuolization in cathepsin null cells as seen by EM analysis, we also found an unusual phenomenon, cell cannibalism (the engulfment of cells by other cells) in cathepsin L -/- cells during cell death induced by CHX or CPT as shown in Fig. 31. We asked whether cathepsins were involved in this process. In order to evaluate the relationship of cathepsin B, D, or L to cannibalism, we used different approaches. First, we explored whether the dying cells were engulfed by live cells or vice versa. Cells were visualized by using *CellTracker* fluorescent dyes, and then cathepsin B, D, or L null cells were exposed to 100 μg/ml CHX for 72 hrs, and WT and MCF-7 cells were exposed to CHX (100 μg/ml) for 48 hrs. At 48 hr-exposure to CHX, similar amount of cell death could be induced in WT and MCF-7 cells as in knockout cells at 72 hr-exposure to CHX. Since cathespin B, D, or L -/- cells are also caspase-3 -/-, we used

MCF-7 cells, which lack caspase-3 activity as control cells to examine if caspase-3 induces cannibalism. As shown in Fig. 32A, after exposure to CHX, different groups of cells were set up for experiments (group #1: co-culture untreated control cells stained red with untreated control cells stained green; group #2: co-culture untreated control cells stained red with CHX-treated cells stained green; group #3: co-culture CHX-treated cells stained red with CHX-treated cells stained green). The cells were analyzed by confocal microscopy. As shown in Fig. 32B, this experiment was able to reveal cells stained in red or green engulfed by cells stained red or green, which validates the model for analyzing which cells (dying cells or live cells) are more cannibalistic. Furthermore, the cannibalized cells were quantitated and the percentage of the cannibalized cells was determined. Cathepsin D, L -/- cells and MCF-7 cells showed increased numbers of cannibalized cells in group #2, in which untreated red cells were cultured with CHX-treated green cells,and group #3, in which CHX-treated red cells were cultured with CHX-treated green cells, by comparing group #1, in which untreated red cells were cultured with untreated green cells.(Fig. 33). In group #2, only cathepsin L -/- cells showed more cannibalized cells (22.8%) than WT cells (12.1%). In group #3, cathepsin D -/- cells showed 19.2% cannibalized cells, cathepsin L -/- cells showed 19.4% cannibalized cell, and MCF-7 cells showed 14.6% cannibalized cell, which is increased by comparing to group #1 (Fig. 33). Comparing with MCF cells, more cannibalized cells were found in group #2 and #3 of cathepsin L -/- cells and in group #3 of cathepsin D -/- cells. This finding suggested that cathepsin D, L and caspase-3 activity play roles in regulation of cannibalism, and more cannibalized cells can be induced in cahtepsin D or L null cells when the cells were exposed to CHX.

Second, to further observe the phagocytic activity in cathepsin null cells during cell death, we used FACS analysis to evaluate the amount of *Saccharomyces cereviside* zymosan A BioParticles conjugated with FITC engulfed by cells during cell death. After the cells were exposed to 50 μM CPT and 100 μg/ml CHX for 48 hrs, cells were incubated with 0.1mg/ml particles for 2 hrs and the percentage of the cells that could phagocytose more particles than controls was determined by FACS analysis according to the intensity of fluorescence. After exposure to CPT, 10.1% WT cells, 30.8% cathepsin B -/- cells, 13.6% cathepsin D -/- cells, 9.5% cathepsin L -/- cells, and 33.5% MCF-7 cells showed greater ability to take up particles (Fig. 34). After exposure to CHX, 13.1% WT cells, 11.8% cathepsin B -/- cells, 17.2% cathepsin D -/- cells, 13.4% cathepsin L -/- cells, and 33.0% cells MCF-7 cells were able to take up more particles (Fig. 34). The cells lacking cathepsin B, D, and caspase-3 showed higher ability than WT cells to engulf small particles during cell death. However, the ability to engulf more small particles in cathepsin L -/- cells is similar to that of WT cells (Fig. 34).

Taken together, the results from different methods showed that some changes that are not characteristic of apoptosis occurred in cathepsin B, D, and L null cells, which may result from the lack of cathepsin B, D, L or caspase-3. Cathepsin D or L may be more important in the regulation of cannibalism.

**C. Examination of expression and activation of Cdk5 during cell death in cathepsin B, D, or L null and wild type cells.**

Having established that cell death can be induced by CHX and CPT in cathepsin B-/-, or cathepsin D-/-, or cathepsin L -/- and wild type cells although with atypical

morphological changes, we asked whether Cdk5 is activated in this type of cell death. All cells were treated with CHX and CPT for 8 hrs, 24 hrs and 72 hrs and the level of Cdk5 expression under these conditions was examined by western blot using anti-Cdk5 antibody. As expected, we found similar levels of Cdk5 expression in both untreated control and CHX or CPT treated cells in both wild type and cathepsin null cells (Fig. 35). The expression of Cdk5 protein is not affected by either cell death or lack of cathepsins. This is consistent with previous data from our laboratory using different models (Ahuja et al. 1997; Zhu et al, 2002; Lin et al 2006) that Cdk5 is a stable protein and the level of Cdk5 expression does not change during induced cell death.

Although the level of Cdk5 expression is not changed in either wild type or cathespin null cells during cell death, its activity may be altered. To answer whether Cdk5 is activated during cell death we examined the generation of p25, the specific activator of Cdk5, since our previous work has shown a direct correlation between p25 production and Cdk5 activation in several models (Zhu et al., 2002; Lin et al., 2006), as well as the phosphorylation of histone H1, a substrate of Cdk5.

Equal amounts of protein from wild type and cathepsin B, D, or L -/- cells treated with CHX and CPT for 72 hrs, at which time massive cell death was induced, were prepared as in Materials and Methods, and separated on a 12.5% SDS-PAGE gel. Western blot analysis showed the appearance of a band at 25 kDa when cell death was induced by CHX and CPT in wild type cells, while in cathepsin B, D, and L -/- cells, p25 production was detected only in CPT-treated cells, but not in CHX-exposed cells (Fig. 36A & B). Thus lack of cathepsin B, D, or L did not affect the generation of p25 during cell death. We also examined the activity of Cdk5 by its ability to phosphorylate histone

H1. We found that Cdk5 kinase was activated in both CHX and CPT-treated wild type cells and CPT-treated cathepsin B, D, or L -/- cells after 72 hrs exposure of CHX and CPT, by the detection of the band of phosphorylated histone H1 at 31kDa (Fig. 36B & D). The results of Cdk5 kinase activity in wild type and cathepsin B, D or L -/- cells indicate that the activation of Cdk5 is not affected by the absence of cathepsin B, or D, or L.

Taken together, our data supported the conclusion that the activation of Cdk5 and induction of p25 production does not require cathepsin B, D, or L function during cell death.

### 3. Discussion

In our study, we induced cell death by CHX or CPT in wild type and cathepsin B -/-, cathepsin D -/- and cathepsin L -/- cells after 72 hrs treatment, suggesting that ultimately CHX or CPT mediated cell death is independent of cathepsin B, cathepsin D and cathepsin L. Since at 24 hrs the level of cell death in cathepsin B, D, or L null cells is much lower than in wild type cells, we further examined the kinetics of cell death induced by CHX or CPT and found that there was a delay in the induction and progression of cell death in cathepsin B, D, or L null cells. Although cell death in cathepsin B, D, or L knockout cells is delayed, cell death is induced by CHX and CPT. Cdk5 is activated and p25 is generated during cell death in cathepsin B, D, or L null cells, indicating that Cdk5 activation during cell death does not require cathepsin B, or D, or L activation. During cell death induced by CHX, we do not see generation of p25 generation. However, Cdk5 is activated at a lower level than that seen during cell death induced by CPT, confirming that p25 generation is not required for Cdk5 activation. The consistent level of Cdk5 protein expression in wild type cells and cathepsin B, or D, or L -/- cells either untreated or treated with CHX or CPT supports the argument that Cdk5 is a relatively stable protein and that Cdk5 activity is not regulated at either transcriptional or translational level, but at a post-translational level as our laboratory has stated previously (Zhu et al., 2002; Lin et al., 2006). The fact that Cdk5 activity is not affected by blockage of lysosomal protease indicates that Cdk5 activity involving in cell death is not downstream of lysosomal proteases.

During the progress of cell death in cathepsin null cells, nuclear fragmentation was inhibited as shown in Hoechst staining although TUNEL assay revealed that fragmented DNA was detected, indicating that functional cathepsins play roles in nuclear fragmentation and that lack of cathepsin B, D, or L can affect the morphological changes in dying cells. Furthermore, EM analysis confirms that the type of cell death induced by CHX and CPT in cathepsin B, D, or L null cells is altered and is atypical by comparison to wild type cells, including vacuolization, formation of autophagic vacuoles, and incomplete nuclear fragmentation suggesting that cathepsin B, D, or L may play roles in the regulation of the progress of cell death and their activities are involved in vacuolization and formation of vacuoles during cell death. Furthermore, by EM we found cell cannibalism in cathepsin L -/- cells. Cell cannibalism is defined as the ability of a cell to phagocytose another cell, a property of malignant tumor cells (DeSimone et al., 1980; Abodief et al., 2006). Our understanding of cannibalism is very limited. New data from Lugini et al., 2006 suggest that cannibalism in melanoma cells is characterized by an overexpression of cathepsin B and that suppression of cathepsin B activity leads to a decrease in cannibalistic activity of metastatic melanoma cells. However, our cathepsin B knockout cells did not decrease or increase cell cannibalism. However, cells lacking of cathepsin D or L showed higher percentage of cell cannibalism than WT cells. Although MCF-7 cells, which lack caspase-3, show similar percentage of cell cannibalism as WT cells, suggesting that caspase-3 may not play important roles in the regulation of cannibalism. We also have shown that cathepsin D, or L null cells have more cannibalized cells than MCF-7 cells. These findings indicate that cathepsin D or L is important to suppress cannibalism during cell death, and cathepsin D or L may be more

important. However, cathepsin L -/- cells did not show high ability to take up small particles, indicating that cannibalism is different from phagocytosis and cell cannibalism may require special signals or signaling pathways, which cathepsin D, or L or caspase-3 may inhibit. After we block the activity of these genes, cell cannibalism can be induced during cell death.

The difference of morphology between cannibalism and typical phagocytosis has been reported. Macrophages surround and engulf the external body through pseudopods (Fais & Malorni 2003), while tumor cannibalism appears a very calm phenomenon through which target cells lay down on the cell membrane and suddenly disappear into the cells (Luqini et al., 2006).

The fact that DNA was fragmented during cell death in both wild type cells and cathepsin B, D, or L null cells without caspase-3 activation indicates that caspase-3 activation is not required for DNA fragmentation, which is consistent with the results we have shown in Chapter IV. However, caspase-7 activation and PARP cleavage have been shown in these cells. In addition, caspase-7 is an executor caspase that is highly similar to caspase-3, and caspase-7 and caspase-3 have similar substrate specificity (Fuentes-Prior & Salvesen, 2004; Degterev et al., 2003). Taken together, the findings suggest that caspase-7 may compensate for lack of caspase-3 during cell death and the effect on morphological changes in dying cells in cathepsin B, D, or L null cells may result from the lack of cathepsin B, D, or L, but not caspase-3 activation.

Taken together, cells lacking cathepsin B, D, or L show atypical apoptotic features during induced cell death and Cdk5 is activated in this situation, suggesting Cdk5 activation does not require cathepsins. Since caspase-3 activation is absent in these

cells, this study also confirmed that Cdk5 activation is caspase-3 independent during cell death.

# CONCLUSION

## Conclusion

As mentioned in the introduction, Cdk5 activity is related to cell death during development (Ahuja et al., 1997; Fu et al., 2002; Rosales et al., 2004; Griffin et al., 2004), in adult homeostatic maintenance of various tissues (Zhang et al., 1997), in neuronal cell death involved in neurodegenerative diseases and neuronal injuries (Rademakers et al., 2005; Cruz & Tsai 2004; Guo 2006), and in nonneuronal cell death (Zhu et al., 2002; Lin et al., 2006; Rosales & Lee, 2006). Furthermore, we and others have shown that Cdk5 is activated by calpain-mediated p25 and p29 production during cell death ( Kusakawa et al., 2000; Tsai et al., 2004) In addition we have shown that activation of Cdk5 is independent of p53, Apaf-1, casapse-9, and caspase-3 in nonneuronal cells (Lin et al., 2006).

In this thesis, we explored the roles of Cdk5 during cell death. First, we expanded the correlation between Cdk5 and cell death to neurons in abnormal developmental mouse embryos induced by cyclophosphamide (CP). Furthermore, we demonstrated that Cdk5 activation during neuronal cell death induced by CP is independent of caspase-3. We also confirmed that inhibition of the production of p25 by calpain inhibitor cannot protect cells from death, and calpain activation and p25 or p29 production is not required for the activation of Cdk5 during cell death. Additionally although Cdk5. activation is correlated with cell death we demonstrated that cell death can occur in the absence of activated Cdk5 and downregulation of Cdk5 activity does not affect the type of cell death. Lastly, we illustrated that Cdk5 activation during cell death is independent of

cathepsin B, or D, and L, and therefore is likely to be independent of the lysosomal cell death pathway altogether.

We find, by several techniques, a direct correlation between the occurrence of cell death and activation of Cdk5. We established that Cdk5 is activated during cell death but that its activation is independent of caspase-3, calpain, cathepsin B, cathepsin D, and cathepsin L as well as p53, Apaf-1, and caspase-9, during cell death. We conclude that there is a general correlation between Cdk5 activity and cell death and that its activation may be upstream of the pathways under influence of these genes. One might assume that Cdk5 would be an essential component of the cell death machinery. However, our findings that the downregulation of Cdk5 activity has no effect on cell death suggest that Cdk5 activity is more likely to be a consequence of cell death than a regulator of it. The biological explanation of why it is so routinely activated is unclear.

Activated Cdk5 plays a role in cell motility, cell adhesion, and neuron migration by phosphorylating different components of the cytoskeleton, such as neurofilaments (Nayeem et al., 2007), actin (Causeret et al., 2007), microtubules (Pandithage et al., 2008), and myosin (Ledee et al, 2007). Furthermore, the microtubule-associated tau protein is a substrate of Cdk5 kinase (Sengupta et al., 2006). Phosphorylation of WAVE1 (Wiskott-Aldrich syndrome protein) by Cdk5 kinase regulates actin polymerization and dendritic spine morphology in mammals (Kim et al., 2006). Therefore, we might postulate a role for Cdk5 kinase in dying cells: that Cdk5 activation results in the phosphorylation of cytoskeletal substrates, altering their assembly. These changes result in the morphological changes in dying cells. The question remains to identify the specific cytoskeletal system that is affected by the activated Cdk5. Additionally since Cdk5 is

activated without p25 and p29 production, one can also postulate that there are other intracellular, yet unidentified, proteins that can activate Cdk5 during cell death.

A further insight achieved by our study has come from the data obtained in cathepsin knockout cells. Although the cathepsins do not seem to be related to, or affect the activity of, Cdk5, cathepsins function in initiation of cell death and the morphological changes developed during cell death since cell death is delayed and nuclear fragmentation is inhibited in cathepsin null cells. Further we find an increase in cell cannibalism in cathepsin null cells, which indicates that cathepsins directly or indirectly regulate cell cannibalism. How and what the relationship is between cathepsins, cell death and cannibalism remains to be explored.

## FIGURES AND FIGURE LEGENDS

**Figure 1. DNA fragmentation in CP-treated caspase-3 +/+ mouse embryos by TUNEL assay.**

Pregnant female mice were injected with CP at gestation day 12.5.The embryos were removed 18 hrs after exposure to CP and sections were prepared as described in Materials and Methods. DNA fragmentation was determined by TUNEL assay.

A, B & C. DNA fragmentation by TUNEL assay showed a few cells with positive signals in caspase-3 +/+ mouse embryo controls (no CP treatment). 400X. A, trigeminal ganglia (TG); B, midbrain (MB); C, future cerebral cortex (CC).

D, E & F. DNA fragmentation by TUNEL assay showed many cells with positive signals in caspase-3 +/+ mouse embryo with CP treatment. 400X. D, TG; E, MB; F, CC.

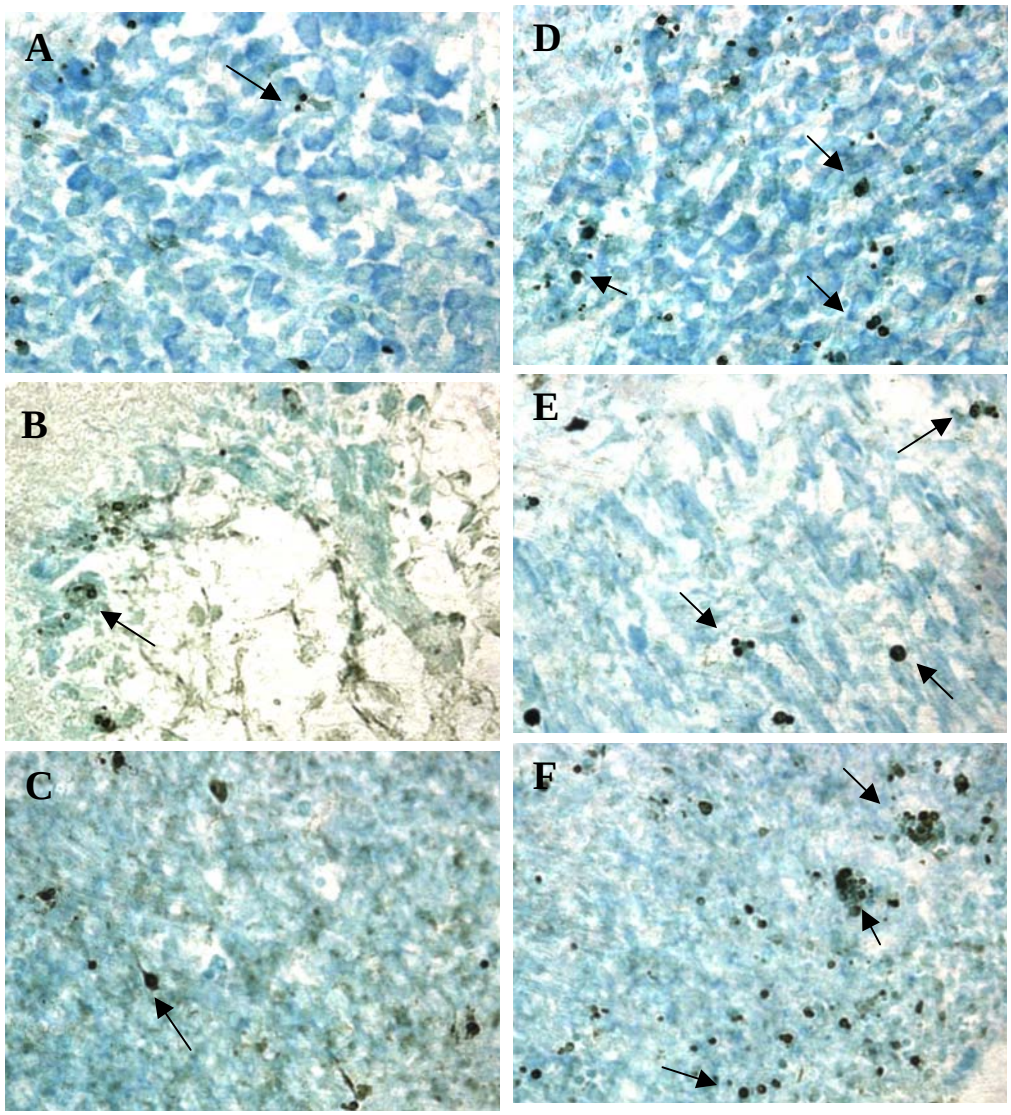


**Figure 1**

**Figure 2. DNA fragmentation in CP-treated caspase-3 -/- mouse embryos by TUNEL assay.**

The preparation was as in Figure 1.

A, B & C. DNA fragmentation by TUNEL assay showed a few cells with positive signals in caspase-3 -/- mouse embryos without CP treatment. 400X. A, trigeminal ganglia (TG); B, midbrain (MB); C, future cerebral cortex (CC).

D, E & F. DNA fragmentation by TUNEL assay showed many cells with positive signals in caspase-3 -/- mouse embryos with CP treatment. 400X. D, TG; E, MB; F, CC.

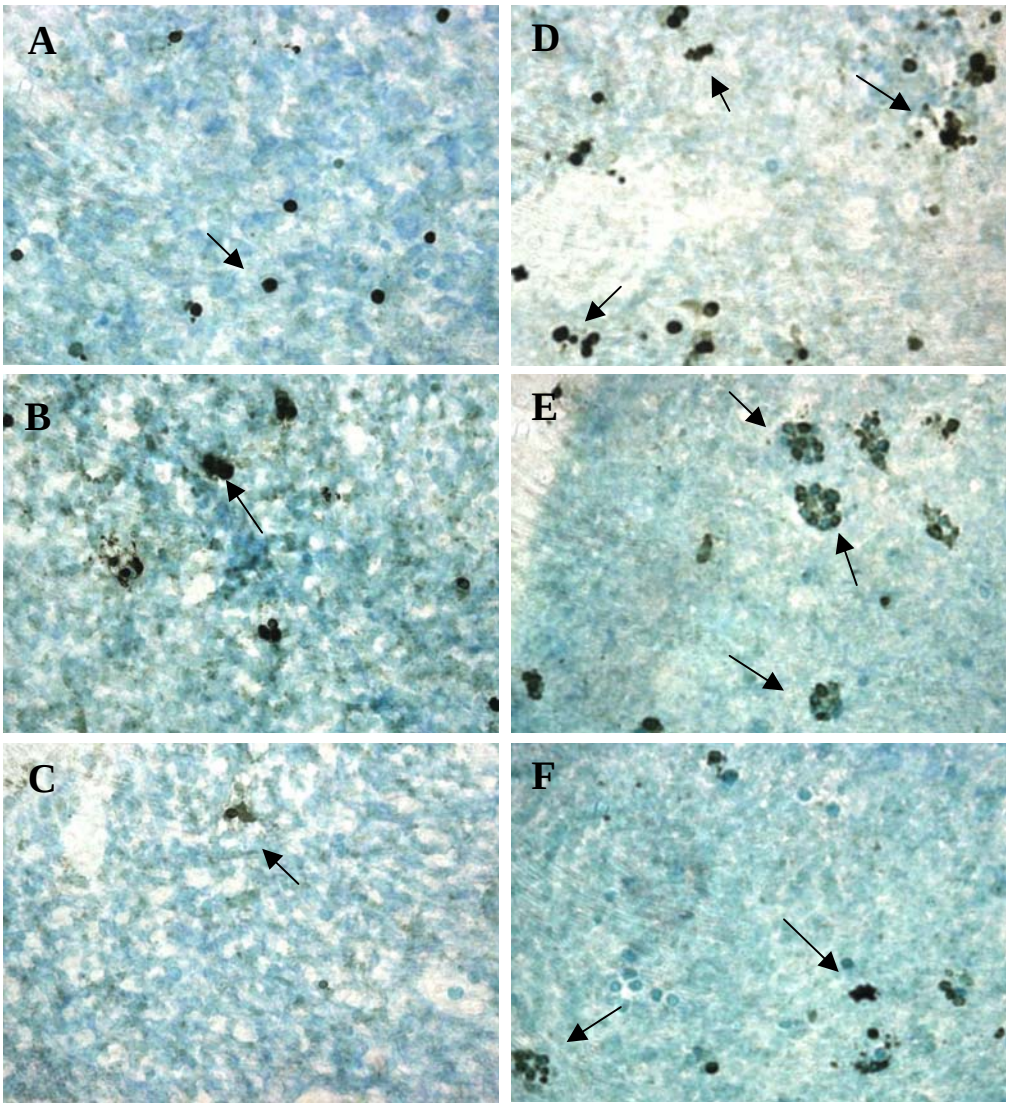


**Figure 2**

**Figure 3. DNA fragmentation is induced by CP, and blockage of caspase-3 activation decreases DNA fragmentation.**

Positive signals were counted in 3-5 fields of (400X) and the number of positive signals per field were used for different plots as shown in A, B, C, and D, and analyzed by t-test. The error bars represent the standard deviation from at least three individual experiments. (* $P < 0.05$)

A. Comparison of the level of DNA fragmentation in different brain regions of caspase-3 +/+ mouse embryos with CP treatment to without CP treatment.

B. Comparison of the level of DNA fragmentation in different brain regions of caspase-3 -/- mouse embryos with CP treatment to without CP treatment.

C. Comparison of the level of DNA fragmentation in different brain regions of caspase-3 +/+ to caspase-3 -/- mouse embryos during CP-induced cell death.

D. Comparison of the level of DNA fragmentation in caspase-3 +/+ and caspase-3 -/- mouse embryos between different brain regions during CP-induced cell death.

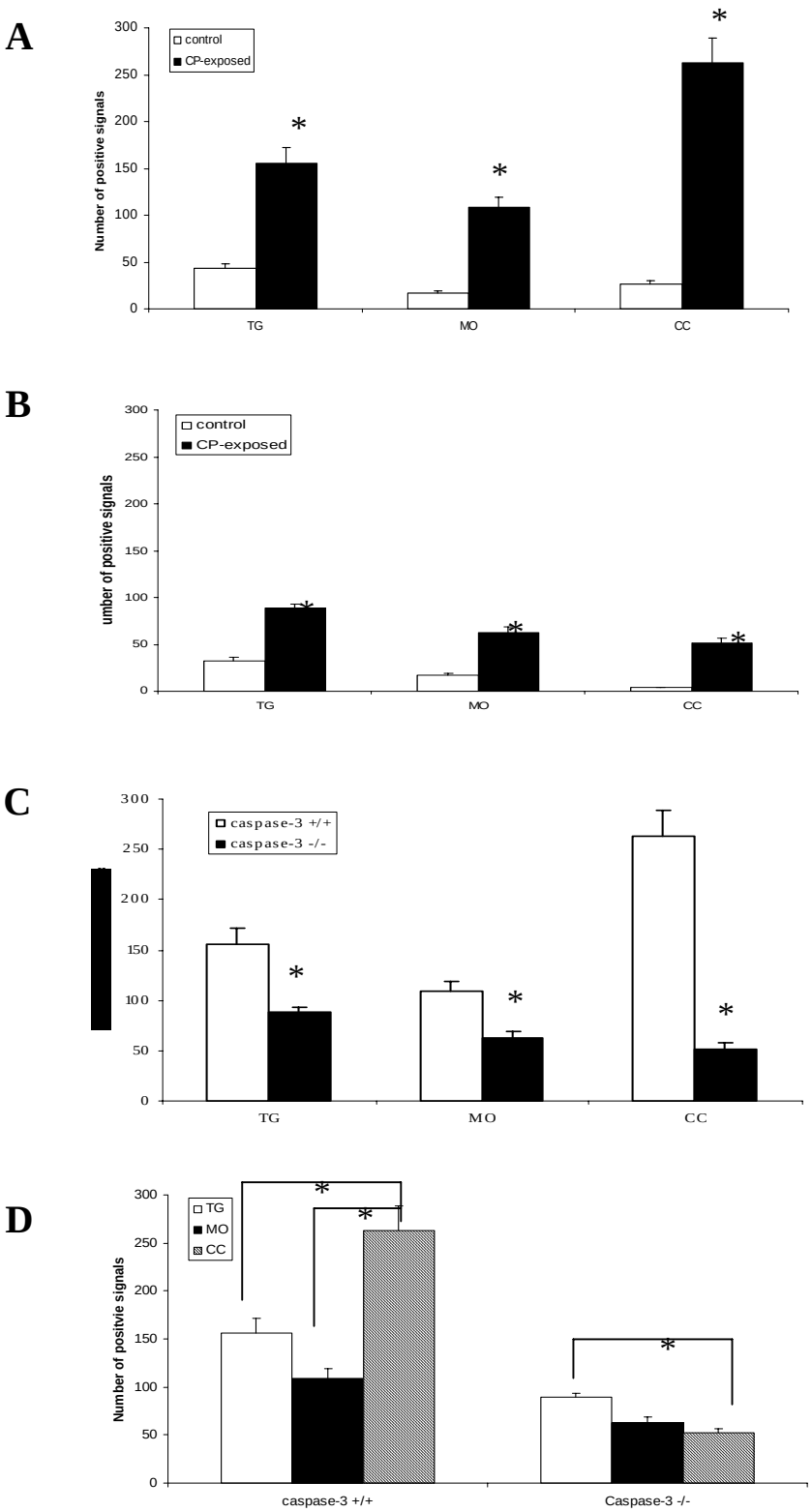
A
control
CP-exposed
Number of positive signals
300
250
200
150
100
50
0
TG
MO
CC
B
control
CP-exposed
umber of positive signals
C
caspase-3 +/+
caspase-3 -/-
D
TG
MO
CC
Number of positivie signals
caspase-3 +/+
Caspase-3 -/-

**Figure 3**

**Figure 4. Caspase-3 activation in CP-treated caspase-3+/+ mouse embryos by immunohistochemistry.**

The preparation was as in Figure 1.

A, B & C. Caspase-3 activation by immunohistochemistry in caspase-3 +/+ mouse embryos without CP treatment showed many cells with caspase-3 activation by anti-active caspase-3 antibody. 400X. A, trigeminal ganglia (TG); B, midbrain (MB); C, future cerebral cortex (CC).

D, E & F. Caspase-3 activation by immunohistochemistry in caspase-3 +/+ mouse embryos with CP treatment showed many cells with caspase-3 activation by anti-active caspase-3 antibody 400X. D,TG; E, MB; F, CC.

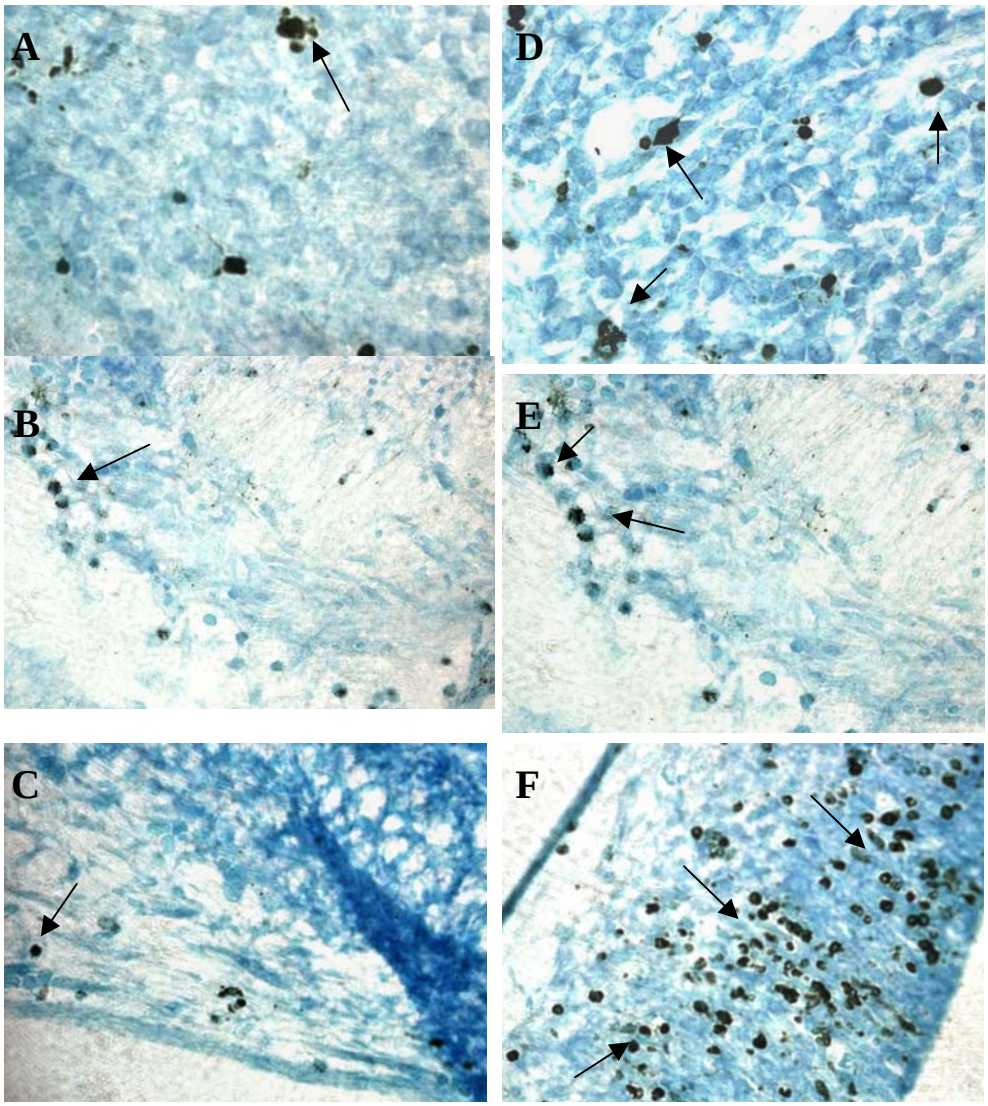


**Figure 4**

**Figure 5. Caspase-3 activation in CP-treated caspase-3 -/- mouse embryos by immunohistochemistry.**

The preparation was as in Figure 1.

A, B & C. Caspase-3 activation by immunohistochemistry in caspase-3 -/- mouse embryos without CP treatment showed no cells with caspase-3 activation by anti-active caspase-3 antibody. 400X. A, trigeminal ganglia (TG); B, midbrain (MB); C, future cerebral cortex (CC).

D, E & F. Caspase-3 activation by immunohistochemistry in caspase-3 -/- mouse embryos with treatment showed no cells with caspase-3 activation by anti-active caspase-3 antibody. 400X. D, TG; E, MB; F, CC.

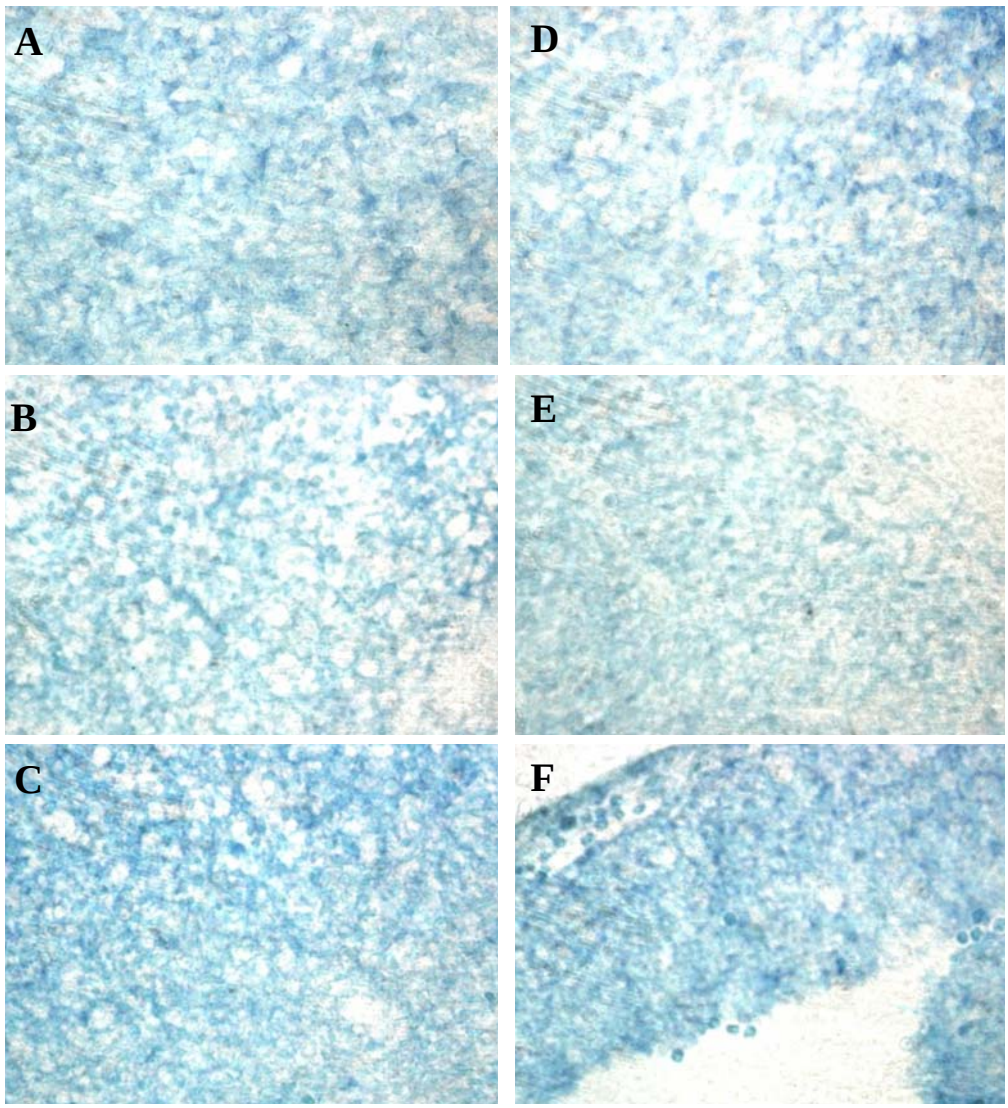


**Figure 5**

**Figure 6. Caspase-3 is activated by CP in caspase-3 +/+ mouse embryos during cell death.**

Positive signals were counted in 3-5 fields of 400X and the number of positive signals per field were used for different plots as shown in A and B, and analyzed by t-test for different plotting. The error bars represent the standard deviation from at least three individual experiments. (* $P < 0.05$)

A. Comparison of the level of caspase-3 activation in different brain regions of caspase-3 +/+ mouse embryos with CP treatment to without CP treatment.

B. Comparison of the level of caspase-3 activation between different brain regions of caspase-3 +/+ mouse embryos during CP-induced cell death.

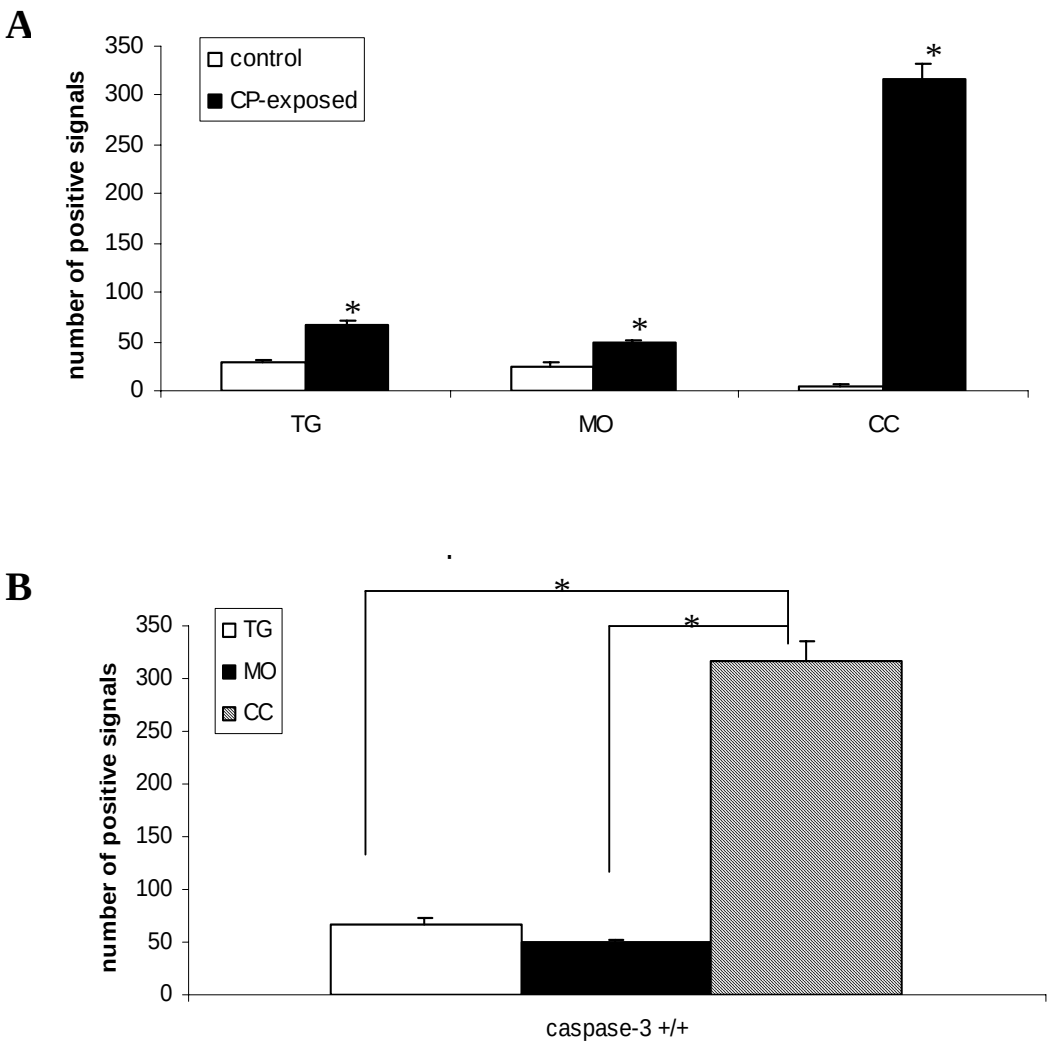
A
control
CP-exposed
number of positive signals
350
300
250
200
150
100
50
0
TG
MO
CC
*
B
TG
MO
CC
number of positive signals
caspase-3 +/+

**Figure 6**

**Figure 7. Cdk5 expression in CP-treated caspase-3 +/+ mouse embryos by immunohistochemistry.**

The preparation was as in Figure 1.

A, B & C. Cdk5 activation by immunohistochemistry showed many cells with Cdk5 activation labeled by Cdk5 antibody in caspase-3 +/+ mouse embryos without CP treatment. 400X. A, trigeminal ganglia (TG); B, midbrain (MB); C, future cerebral cortex (CC).

D, E & F. Cdk5 activation by immunohistochemistry showed many cells with Cdk5 activation labeled by Cdk5 antibody in caspase-3 +/+ mouse embryos with CP treatment. 400X. D, TG; E, MB; F, CC.

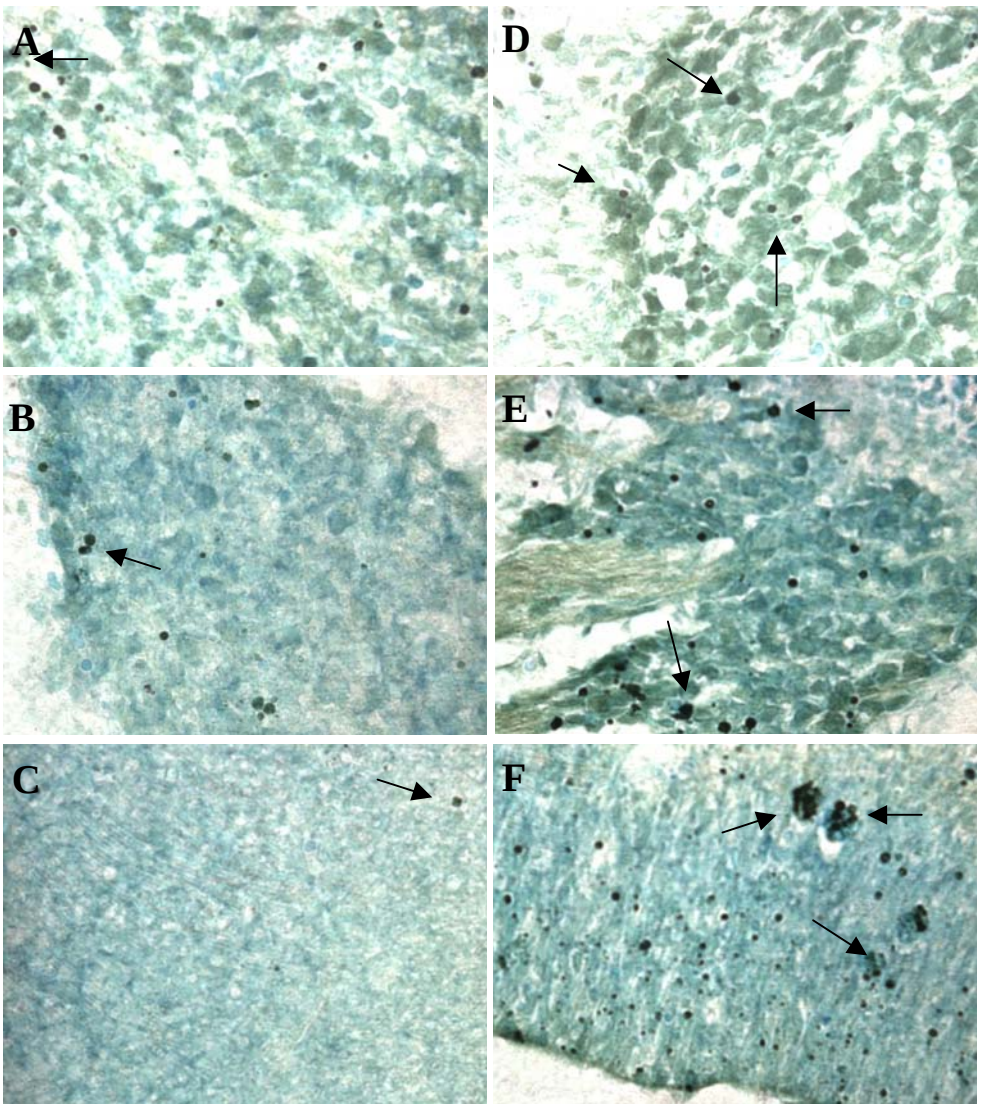


**Figure 7**

**Figure 8. Cdk5 expression in CP-treated caspase-3 -/-mouse embryos by immunohistochemistry.**

The preparation was as in Figure1.

A, B & C. Cdk5 activation by immunohistochemistry showed many cells with Cdk5 activation labeled by Cdk5 antibody in caspase-3 -/- mouse embryos without CP treatment. 400X. A, trigeminal ganglia (TG); B, midbrain (MB); C, future cerebral cortex (CC).

D, E & F. Cdk5 activation by immunohistochemistry showed many cells with Cdk5 activation labeled by Cdk5 antibody in caspase-3 -/- mouse embryos with CP treatment. 400X. D, TG; E, MB; F, CC.

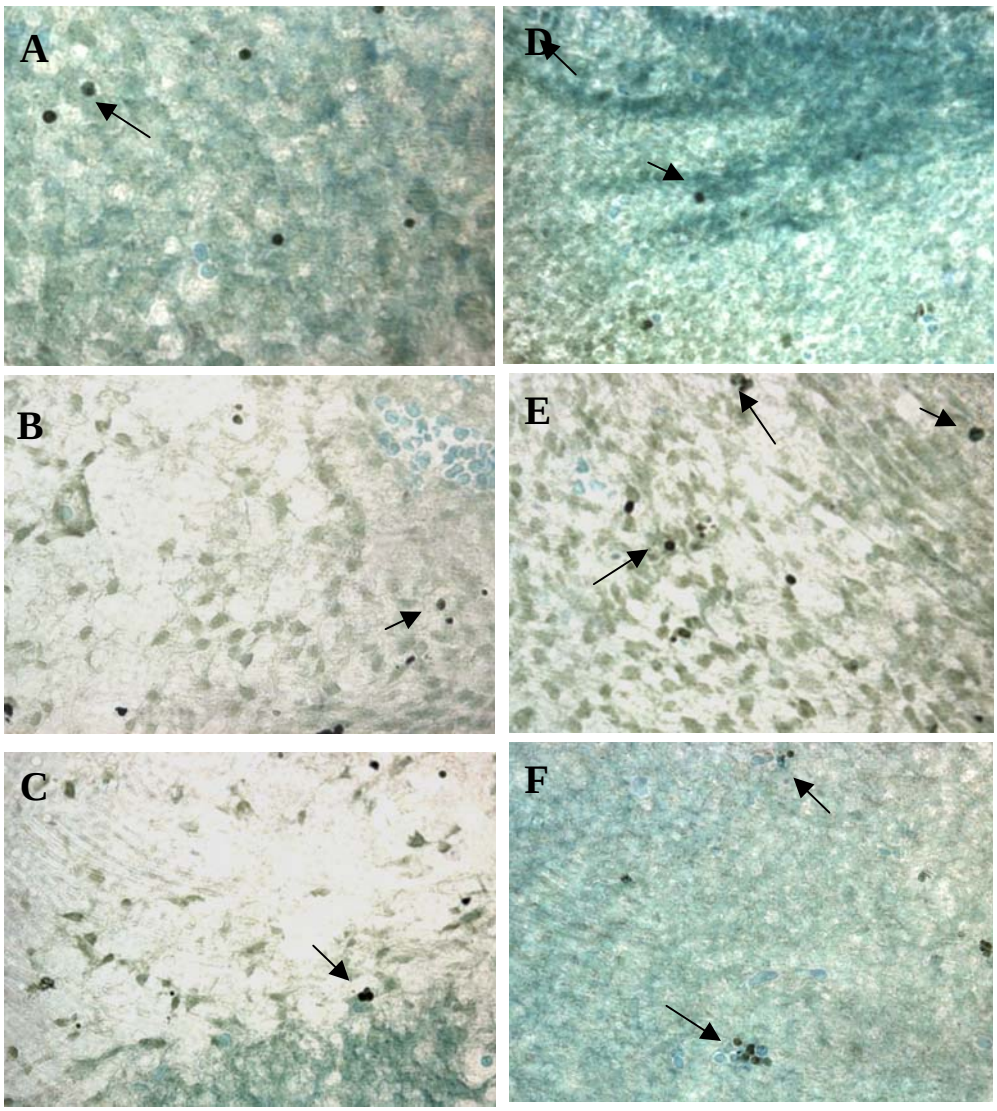


**Figure 8**

**Figure 9. Cdk5 activation is induced by CP, and decreased in caspase-3 -/- mouse.**

Positive signals were counted in 3-5 fields of 400X and the number of positive signals per field were used for different plots as shown in A, B, C, and D, and analyzed by t-test for different plotting. The error bars represent the standard deviation from at least three individual experiments. (* $P < 0.05$)A. Comparison of the level of Cdk5 activation in different brain regions of caspase-3 +/+ mouse embryos with CP treatment to without CP treatment.

B. Comparison of the level of Cdk5 activation in different brain regions of caspase-3 -/- mouse embryos with CP treatment to without CP treatment.

C. Comparison of the level of Cdk5 activation in different brain regions of caspase-3 +/+ to caspase-3 -/- mouse embryos during CP-induced cell death.

D. Comparison of the level of Cdk5 activation in both caspase-3 +/+ and caspase-3 -/- mouse embryos between different brain regions during CP-induced cell death.

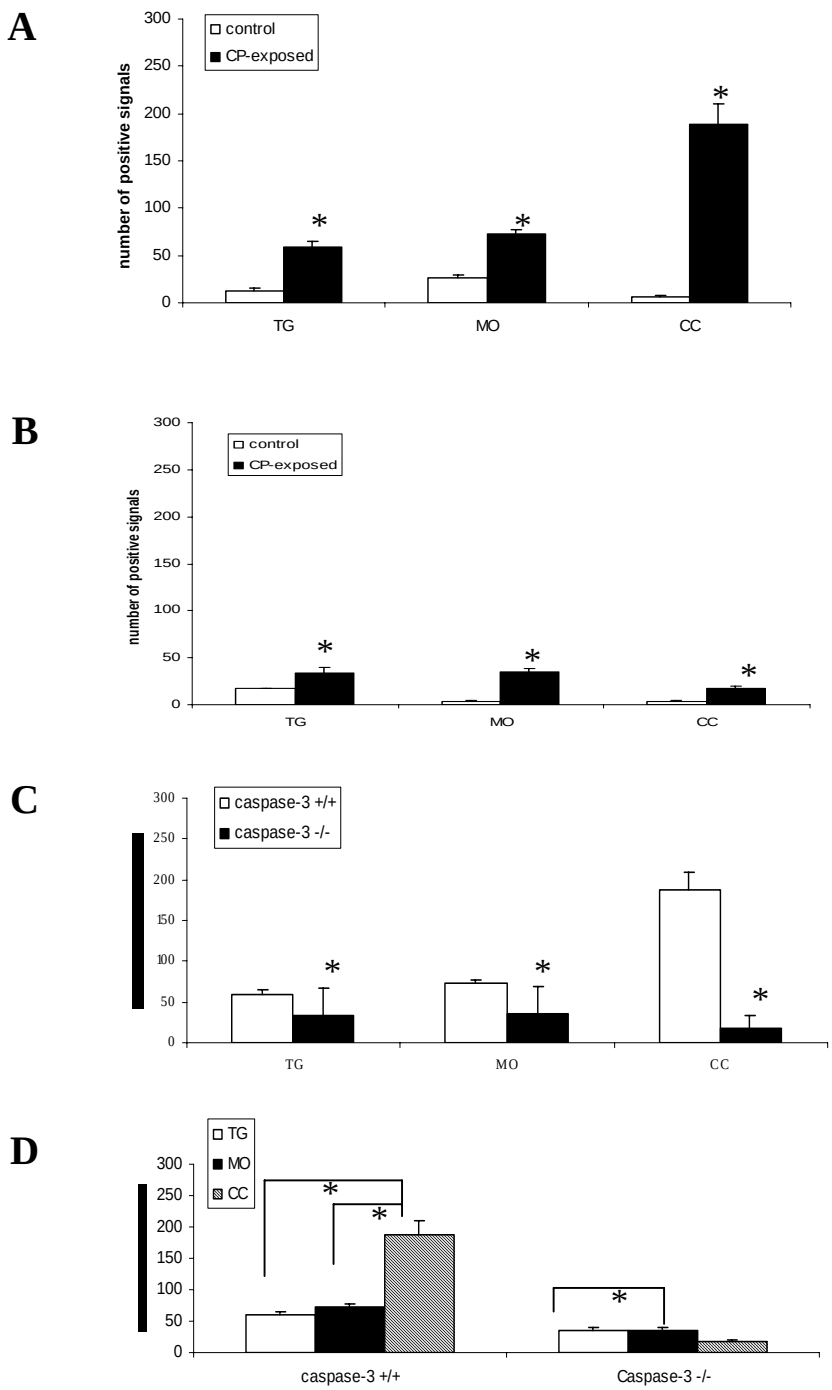
A
control
CP-exposed
number of positive signals
300
250
200
150
100
50
0
TG
MO
CC
B
control
CP-exposed
number of positive signals
TG
MO
CC
C
caspase-3 +/+
caspase-3 -/-
TG
MO
CC
D
TG
MO
CC
caspase-3 +/+
Caspase-3 -/-

**Figure 9**

**Figure 10. Cell death is induced by CPT in C8 cells (Mouse Embryo Fibroblast cells) whether or not calpain is active.**

C8 cells were treated with CPT (50 μM) in the presence or absence of calpain inhibitor, PD (15 μM) for 8 hrs and 18 hrs. The amount of cell death was measured by trypan blue exclusion. Cell death was induced by CPT in the presence or absence of calpain inhibitor,PD. The error bars represent the standard deviation from at least three individual experiments.

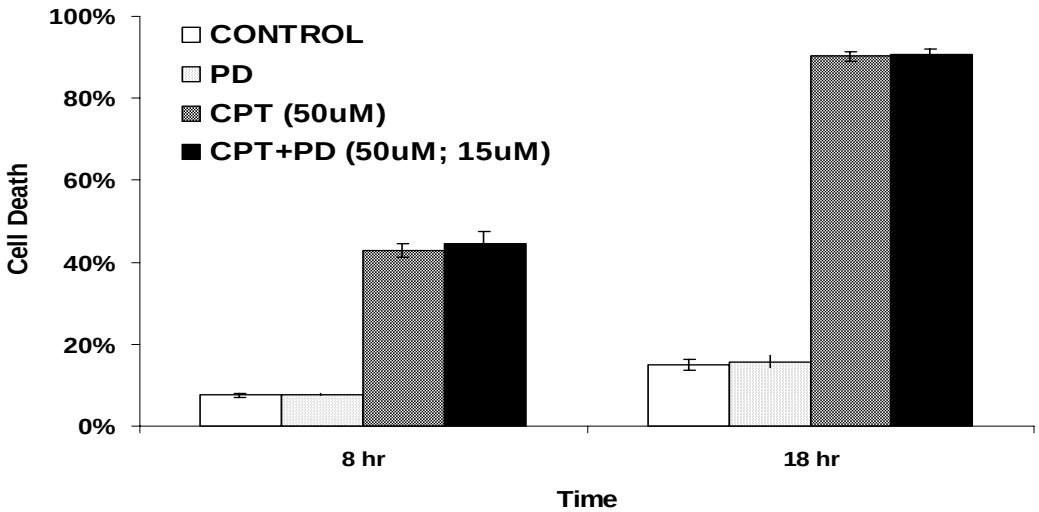
100%
80%
60%
40%
20%
0%
Cell Death
CONTROL
PD
CPT (50uM)
CPT+PD (50uM; 15uM)
8 hr
18 hr
Time

**Figure 10**

**Figure 11. Expression and activation of Cdk5 in C8 cells during CPT-induced cell death in the presence or absence of PD.**

Cells treated with CPT (50 μM) in the presence or absence of PD (15 μM) were collected and lysed by RIPA buffer.

A & B. Histone H1 kinase activity of Cdk5 immunoprecipitates was measured during induced cell death. Following 8 hrs (A) or 18 hrs (B) treatment with CPT in the presence or absence of PD, kinase activity of Cdk5 immunoprecipitaes was determined using histone H1as i*n vitro* substrate. The histone H1 phosphorylated by Cdk5 was detected by western blot using anti-phospho-histone H1 antibody. Cells induced to die by CPT in the presence or absence of PD showed different levels of kinase activity corresponding to cell death.

C. Western blot analysis of equal amount of cell lysates from cells treated with CPT in the presence or absence of PD for 18 hrs using Cdk5 antibody showed an unchanged level of Cdk5 protein during cell death.

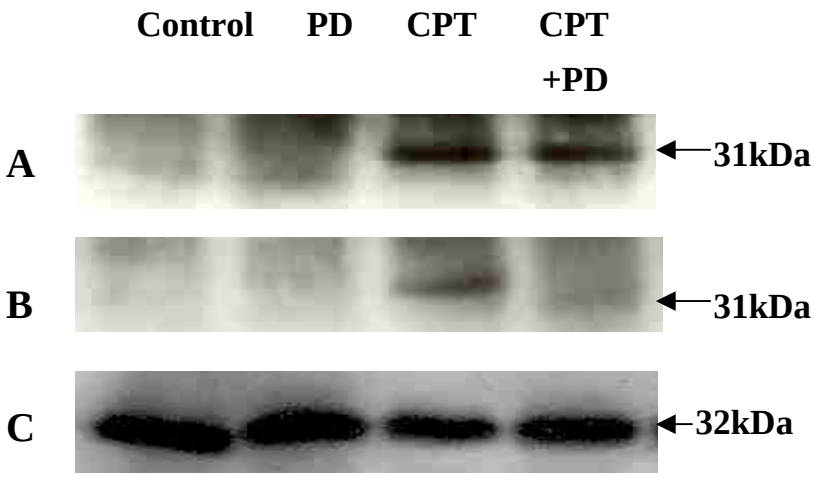


**Figure 11**

**Figure 12. Nuclear fragmentation during CPT-induced cell death by Hoechst Staining in the presence or absence of PD in C8 cells.**

A. B, C & D. C8 cells treated with CPT with Hoechst fluorescent dye, which specifically binds to the nucleus. Fluorescence microscopy showed the changes of nuclei during induced cell death. Untreated control cells (A); cells incubated with PD (B); CPT-treated cells (C); CPT-treated cells incubated with PD (D). Condensation and fragmentation of nucleus indicates apoptosis.

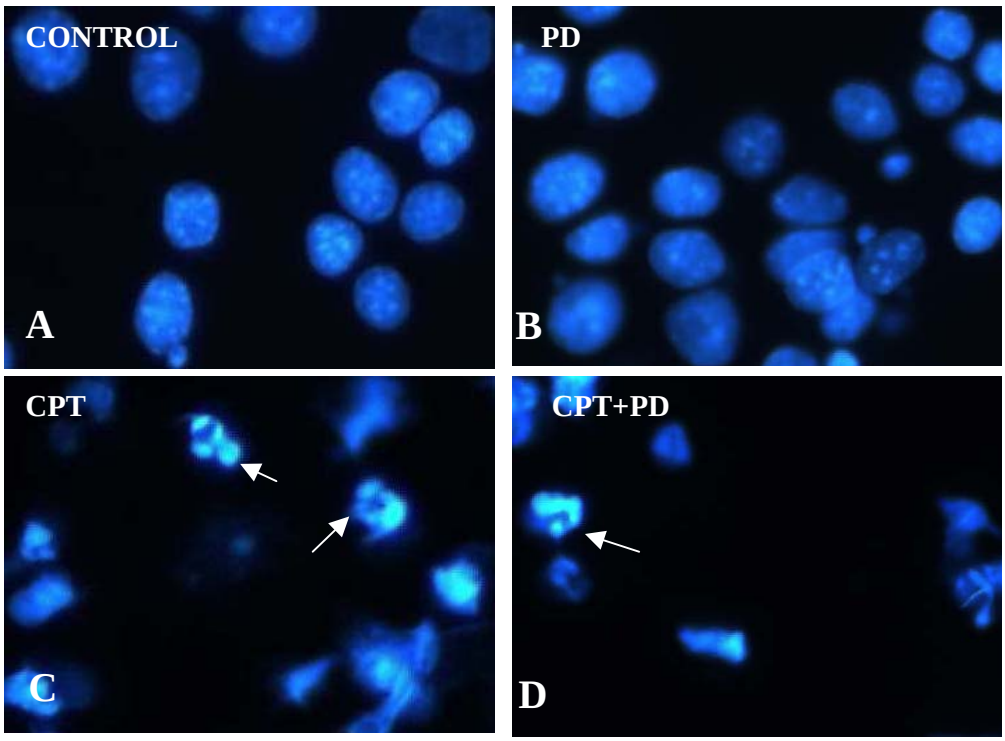


**Figure 12**

**Figure 13. Caspase-3 activation during CPT-induced cell death in the presence or absence of PD.**

A. Western blot analysis of equal amounts of protein samples from C8 cells treated with CPT in the presence or absence of PD for 8 hrs and 18 hrs using anti-active caspase-3 antibody showed the induction of the active caspase-3 by CPT both in the presence and in the absence of PD.

B. Western blot analysis of β-tubulin expression demonstrated equivalent loading of protein samples.

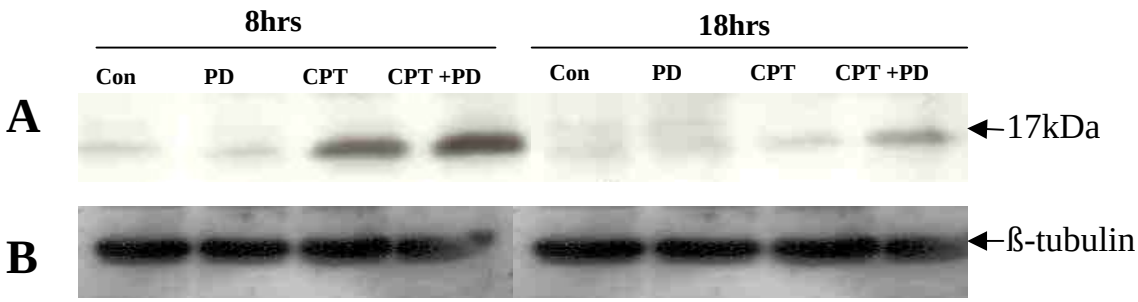
8hrs
18hrs
Con
PD
CPT
CPT +PD
Con
PD
CPT
CPT +PD
A
17kDa
B
ß-tubulin

**Figure 13**

**Figure 14. Morphological analysis of cell death in C8 cells by electron microscopy.**

As described in Materials and Methods, C8 cells treated with CPT at 18 hrs in presence or absence of PD were fixed for electron microscopy.

A & B. Untreated control wild type cells were elongated and displayed large normal nucleus.

C & D. C8 cells treated with CPT in the absence of PD showed features of apoptosis as well as vacuolization after 8 hrs treatment and secondary necrosis in more advanced dying cells after 18 hrs treatment. An apoptotic cell has a condensed nucleus and cytoplasm and increased electron density while the chromatin is fragmented. A necrotic cell has disintegrated membranes and nucleus while the cytoplasm is not electron dense.

E & F. C8 cells treated with CPT in the presence of PD showed features of apoptosis as well as vacuolization after 8 hrs treatment and secondary necrosis in more advanced dying cells after 18 hrs treatment. An apoptotic cell has a condensed nucleus and cytoplasm and increased electron density while the chromatin is fragmented. A necrotic cell has disintegrated membrane and nucleus while the cytoplasm is not electron dense.

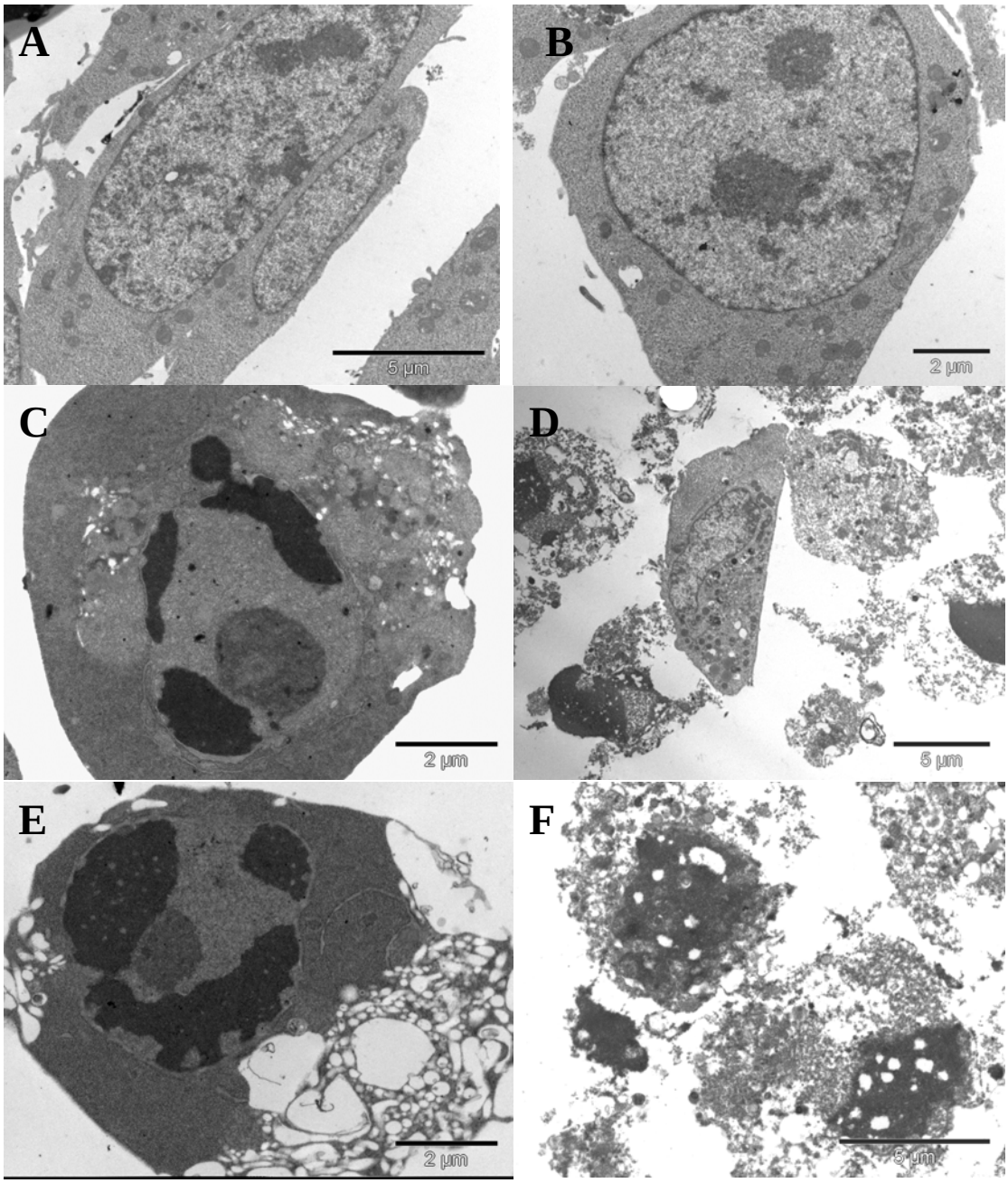


**Figure 14**

**Figure 15. Cell death is induced by CPT when Cdk5 expression is inhibited by RNAi.**

A. COS-7 cells transfected with dsRNA (Cdk5-1, Cdk5-2, Cdk5-3, Cdk5-4, and Cdk5-5) were collected and lysed by RIPA buffer. Western blot of equal amounts of cell lysates from COS-7 cells, using Cdk5 antibody, showed Cdk5 expression in all cells (a) and the level of Cdk5 expression was measured by densitometry (b). dsRNA of Cdk5-1 and Cdk5-3 downregulated Cdk5 expression.

B. COS-7 cells were treated with CPT (25 μM) for 24 hrs and 48 hrs whether or not they were transfected with dsRNA (Cdk5-1, Cdk5-2, and Cdk5-3) . The amount of cell death was measured by trypan blue exclusion. Transfection of dsRNA increased the amount of cell death, and the amount of cell death was not affected by the downregulation of Cdk5 by RNAi. The error bars represent the standard deviation from at least three individual experiments.

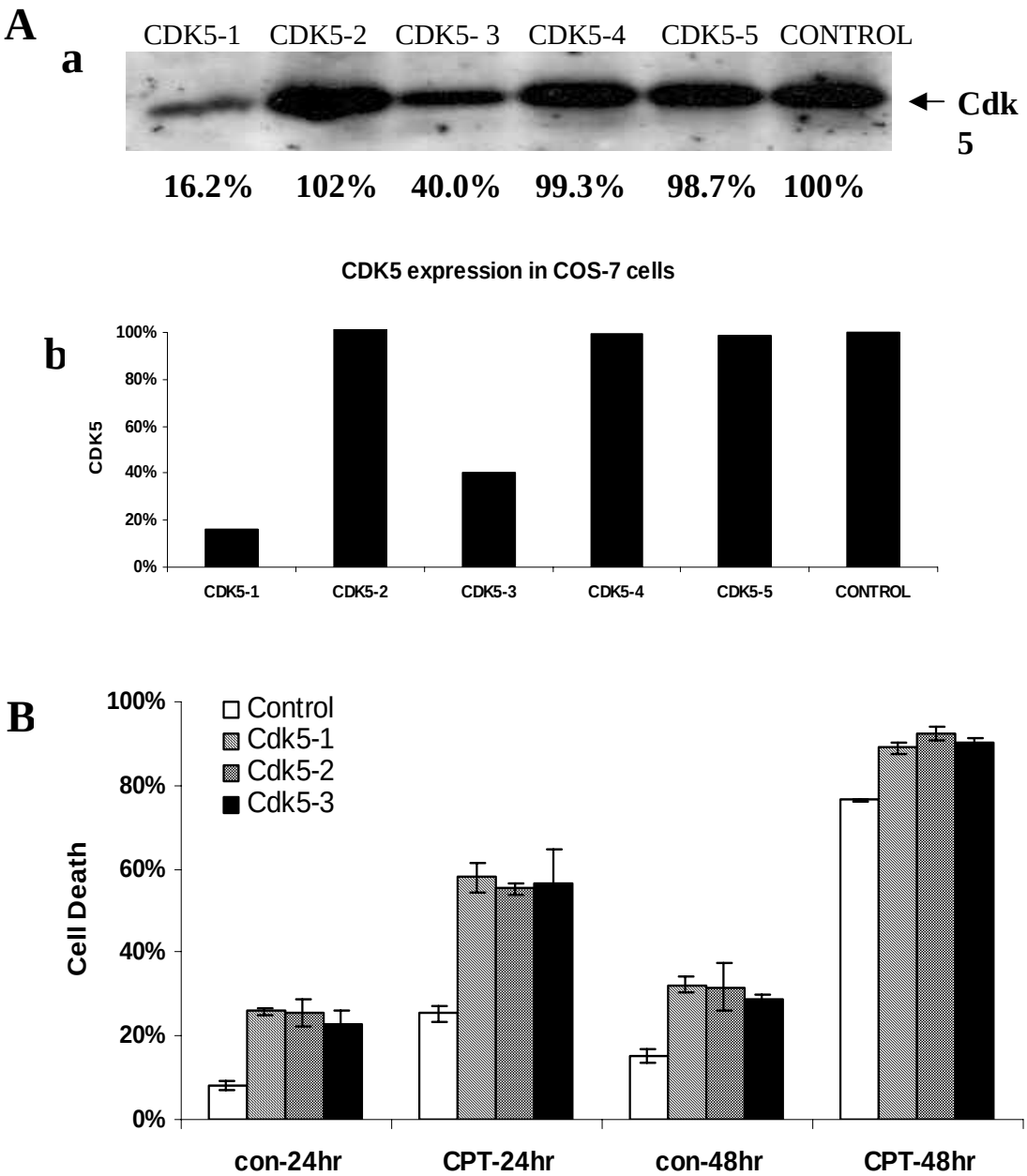
A
a
CDK5-1 CDK5-2 CDK5- 3 CDK5-4 CDK5-5 CONTROL
Cdk 5
16.2% 102% 40.0% 99.3% 98.7% 100%
CDK5 expression in COS-7 cells
b
CDK5
100%
80%
60%
40%
20%
0%
CDK5-1 CDK5-2 CDK5-3 CDK5-4 CDK5-5 CONTROL
B
Control
Cdk5-1
Cdk5-2
Cdk5-3
Cell Death
100%
80%
60%
40%
20%
0%
con-24hr CPT-24hr con-48hr CPT-48hr

**Figure 15**

**Figure 16. Nuclear fragmentation detected by Hoechst staining during CPT-induced cell death when Cdk5 activity is inhibited.**

COS-7 cells treated with CPT (25 μM) were fixed with 4% paraformaldehyde and stained with Hoechst fluorescent dye, which specifically binds to the nucleus. Hoechst staining exhibited condensed and fragmented nuclei in dying cells. Untreated and nontransfected control cells (A); CPT-treated and nontransfected cells (B); Untreated Cdk5-1 transfected cells (C); CPT-treated and Cdk5-1 transfected cells (D); Untreated Cdk5-2 transfected cells (E); CPT-treated and Cdk5-2 transfected cells (F). Condensation and fragmentation of nucleus indicates apoptosis.

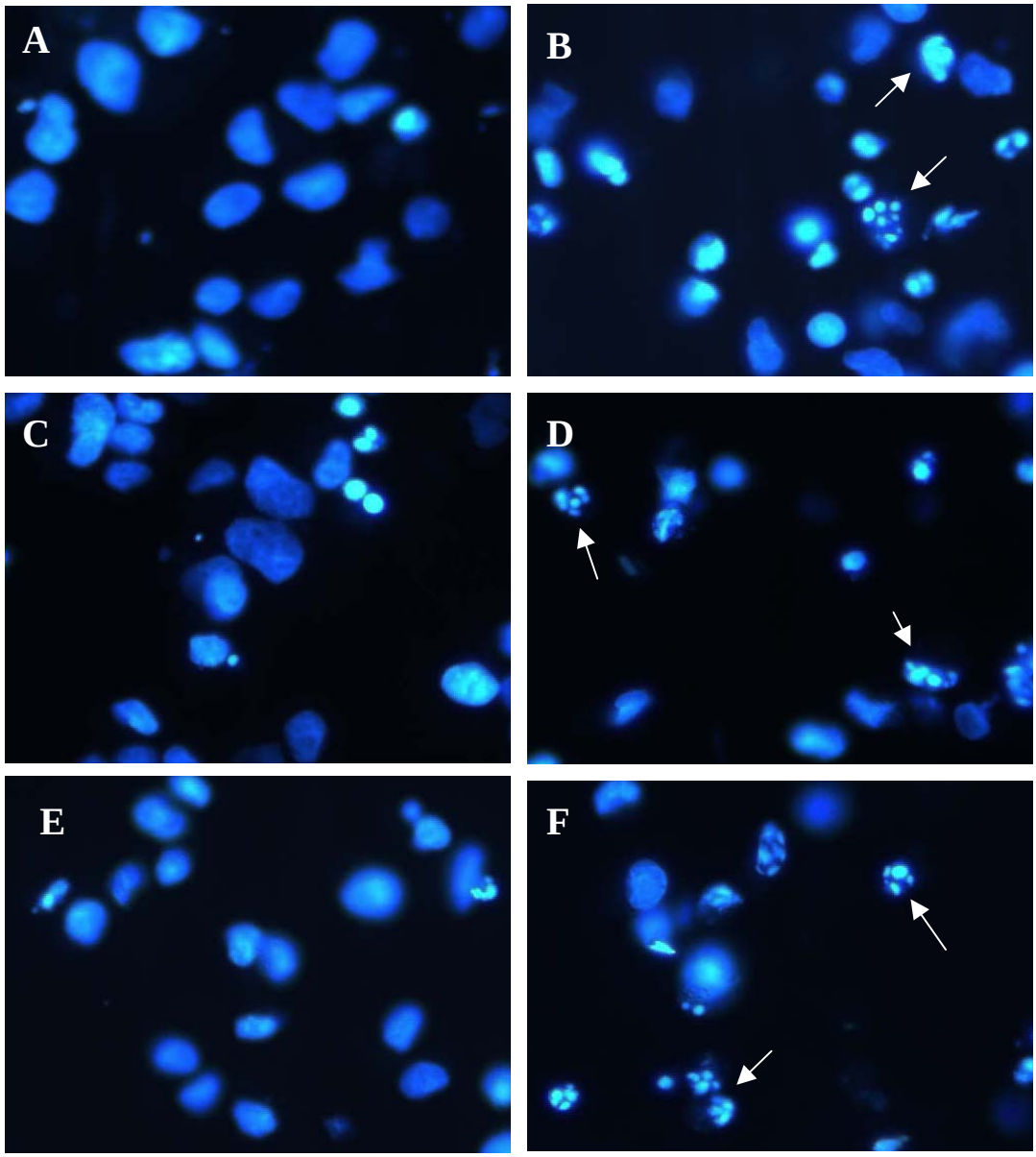


**Figure 16**

**Figure 17. Caspase-3 activation during CPT-induced cell death when Cdk5 expression is inhibited by RNAi.**

COS-7 cells were incubated with CPT (25 μM) for 48 hrs in the presence or absence of dsRNA (Cdk5-1, Cdk5-2 and Cdk5-3). After 48 hrs incubation, cells were collected and proteins were extracted. Western blot analysis of equal amounts of cell lysates from cells using anti-active caspase-3 antibody showed the induction of the active caspase-3 by CPT both in the non-transfected control cells and transfected cells.

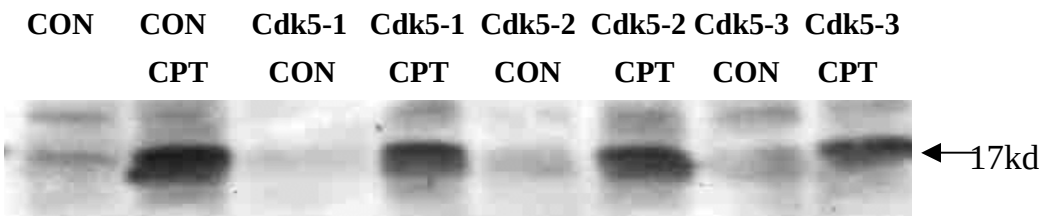
CON
CON CPT
Cdk5-1 CON
Cdk5-1 CPT
Cdk5-2 CON
Cdk5-2 CPT
Cdk5-3 CON
Cdk5-3 CPT
17kd

**Figure 17**

**Figure 18. Morphological analysis of cell death in COS-7 cells by electron microscopy.**

COS-7 cells treated with CPT at 48 hrs with or without transfection of Cdk5 dsRNA were fixed for electron microscopy.

A & B. Untreated cells transfected with Cdk5-1 (A) and Cdk5-2 (B) were elongated and displayed large normal nucleus.

C & D. COS-7 cells treated with CPT in the absence of dsRNA of Cdk5 (no transfection, C) and transfected with dsRNA of Cdk5-1 (D) showed features of apoptosis, such as a condensed nucleus and cytoplasm and increased electron density while the chromatin is fragmented, indicating that cells underwent apoptosis.

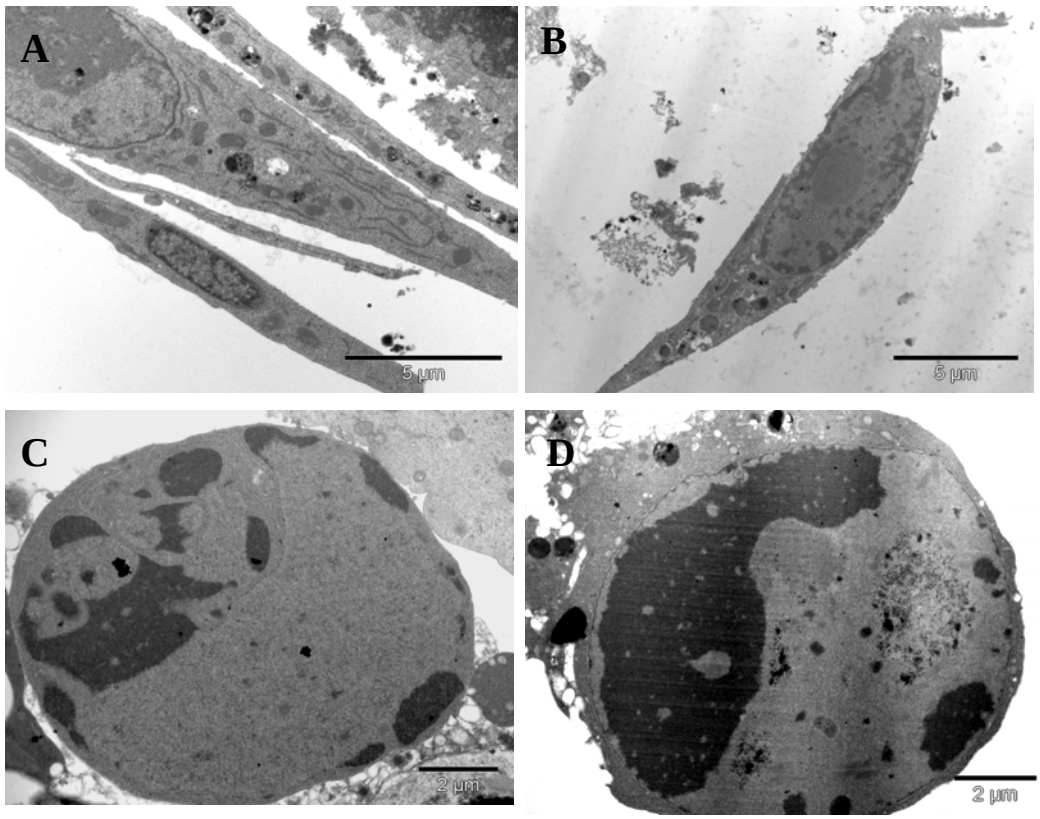


**Figure 18**

**Figure 19. Cell death is delayed in cathepsin B -/-, cathepsin D -/- and cathepsin L -/- cells**.

2.2 X 106 cells were plated in growth media DMEM with 10% FBS and switched to media supplemented with 1% FBS for treatment when the cells reach 80% confluence. Cells were then exposed to different death inducers, camptothecin (CPT, 50 μM) or cycloheximide (CHX, 100 μg/ml). The level of cell death was determined at different times by trypan blue exclusion. The error bars represent standard deviation obtained from at least 3 independent experiments. The amount of the induction of cell death was calculated by subtracting the amount of cell death in untreated control cells.

A. Kinetics of cell death induced by CHX in wild type and cathepsin B -/-, cathepsin D -/- and cathepsin L -/- cells showed that knockout of any one of these proteases delayed the inducation of cell death.

B. Kinetics of cell death induced by CPT in wild type and cathepsin B -/-, cathepsin D -/- and cathepsin L -/- cells. As was the case for CHX, knockout of these proteases delayed cell death induced by CPT.

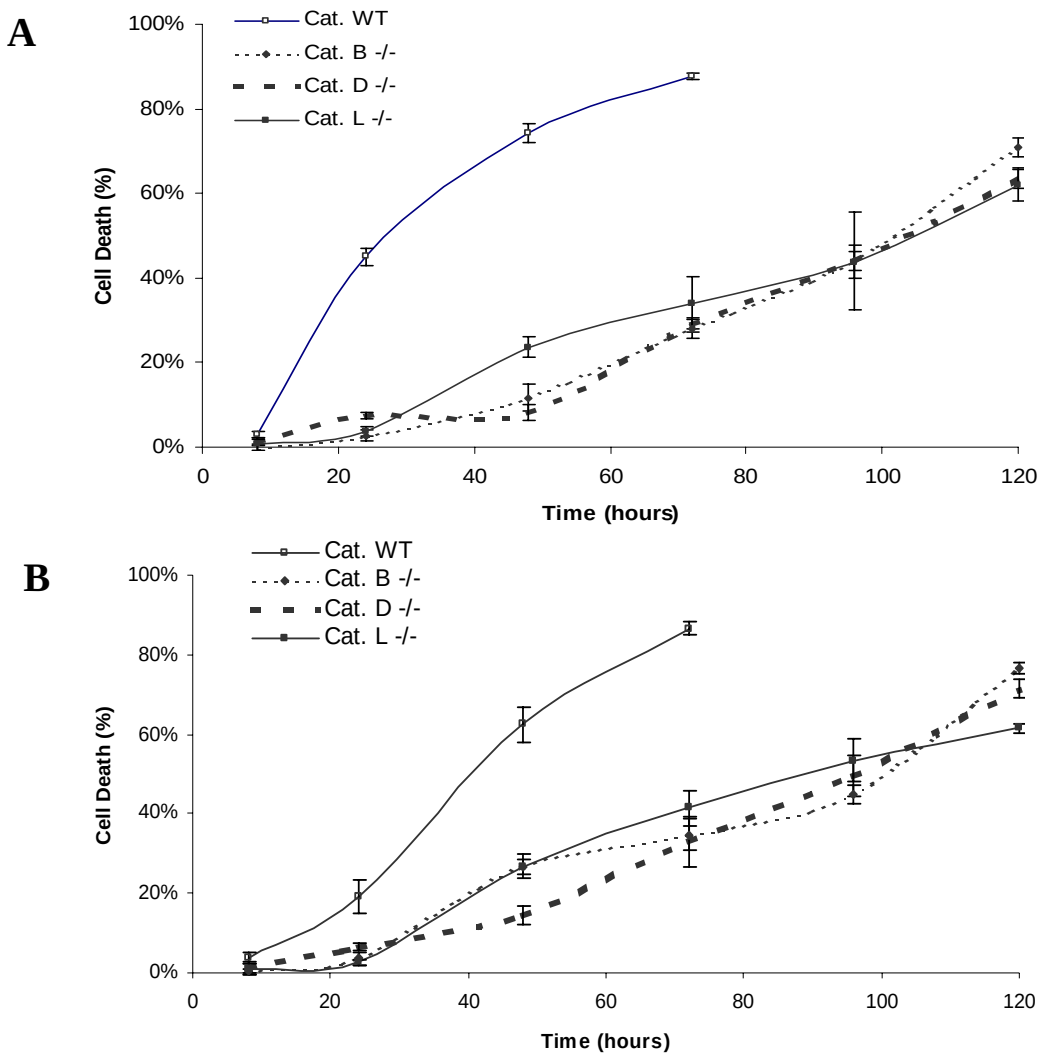
A
Cat. WT
Cat. B -/-
Cat. D -/-
Cat. L -/-
Cell Death (%)
100%
80%
60%
40%
20%
0%
0
20
40
60
80
100
120
Time (hours)
B
Cat. WT
Cat. B -/-
Cat. D -/-
Cat. L -/-
Cell Death (%)
100%
80%
60%
40%
20%
0%
0
20
40
60
80
100
120
Time (hours)

**Figure 19**

**Figure 20. DNA fragmentation during cell death induced by CPT and CHX in wild type cells by TUNEL assay.**

Wild type cells grew on coverslips in DMEM with 10% FBS. After the cells reached 80% confluence, cells were treated with CPT (50 μM) and CHX (100 μg/ml) in DMEM with 1% FBS for 24 and 72 hrs. Wild type cells were fixed with 3% paraformaldehyde, and DNA fragmentation was determined by TUNEL assay as described in Materials and Methods. Dark-brown positive TUNEL signals showed fragmented DNA in CPT-treated for 24 (C) and 72hrs (D) and CHX-treated for 24 (E) and 72 hrs (F) cells, compared with untreated control cells for 24 (A) and 72hrs (B).

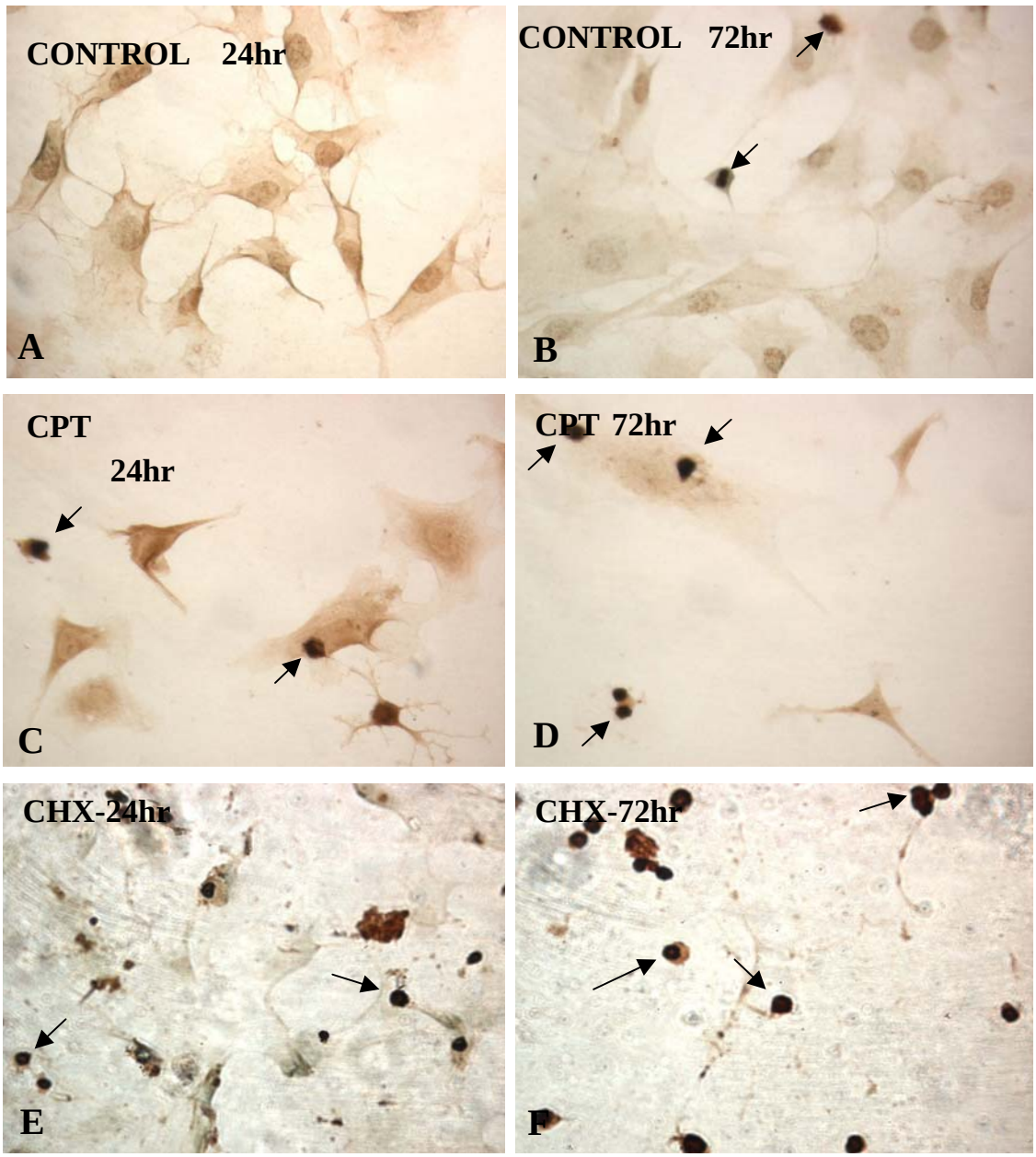
CONTROL 24hr
A
CONTROL 72hr
B
CPT
24hr
C
CPT 72hr
D
CHX-24hr
E
CHX-72hr
F

**Figure 20**

**Figure 21. DNA fragmentation during cell death induced by CPT and CHX in cathepsin B -/- cells by TUNEL assay.**

As described in Figure 20, cathepsin B -/- cells were fixed with 3% paraformaldehyde after 24 and 72 hrs exposure to CPT (50 μM) and CHX (100 μg/ml), and DNA fragmentation was determined by TUNEL assay as described in Materials and Methods. Dark-brown positive TUNEL signals showed fragmented DNA in CPT-treated for 24 (C) and 72 hrs (D) and CHX-treated for 24 (E) and 72 hrs (F) cells, compared with untreated control cells for 24 (A) and 72 hrs (B).

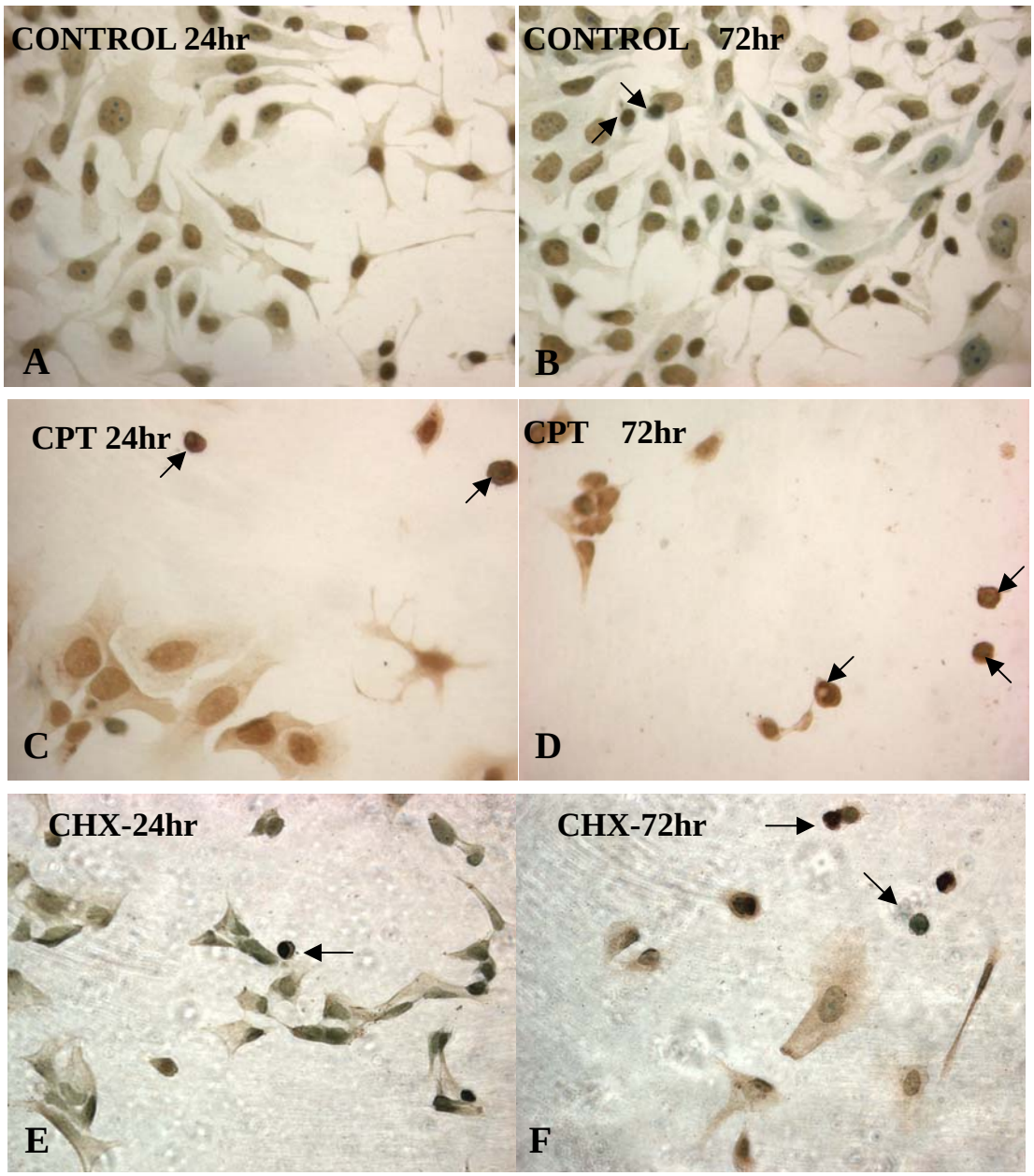
CONTROL 24hr
A
CONTROL 72hr
B
CPT 24hr
C
CPT 72hr
D
CHX-24hr
E
CHX-72hr
F

**Figure 21**

**Figure 22. DNA fragmentation during cell death induced by CPT and CHX in cathepsin D -/- cells by TUNEL assay.**

As described in Figure 20, cathepsin D -/- cells were fixed with 3% paraformaldehyde after 24 and 72 hrs exposure to CPT (50 μM) and CHX (100 μg/ml), and DNA fragmentation was determined by TUNEL assay as described in Materials and Methods. Dark-brown positive TUNEL signals showed fragmented DNA in CPT-treated for 24 (C) and 72 hrs (D) and CHX-treated for 24 (E) and 72 hrs (F) cells, compared with untreated control cells for 24 (A) and 72 hrs (B).

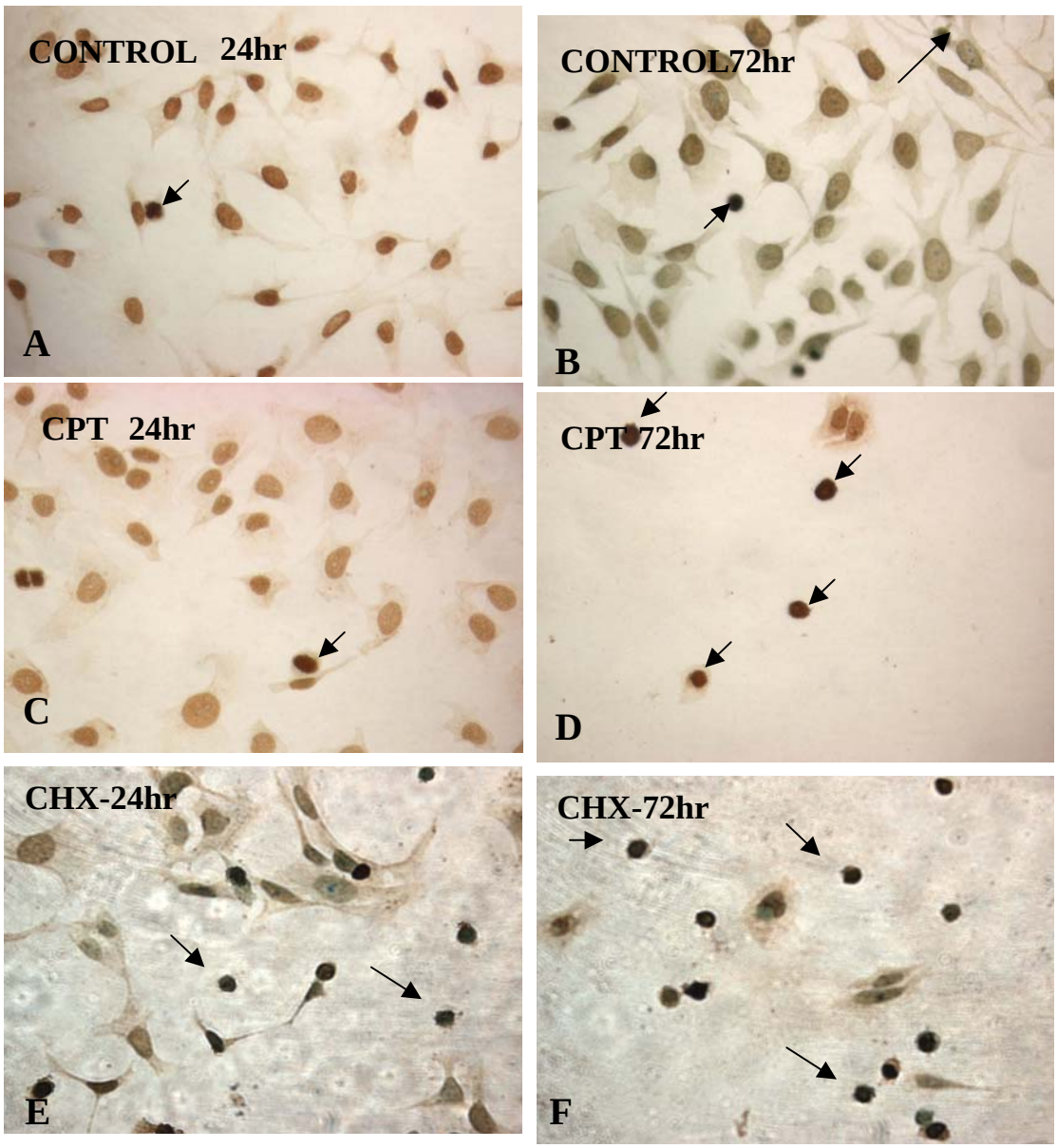
CONTROL 24hr
A
CONTROL72hr
B
CPT 24hr
C
CPT 72hr
D
CHX-24hr
E
CHX-72hr
F

**Figure 22**

**Figure 23. DNA fragmentation during cell death induced by CPT and CHX in cathepsin L -/- cells by TUNEL assay.**

As described in Figure 20, cathepsin L -/- cells were fixed with 3% paraformaldehyde after 24 and 72 hrs exposure to CPT (50 μM) and CHX (100 μg/ml), and DNA fragmentation was determined by TUNEL assay as described in Materials and Methods. Dark-brown color positive TUNEL signals showed fragmented DNA in CPT-treated for 24 (C) and 72 hrs (D) and CHX-treated for 24 (E) and 72 hrs (F) cells, compared with untreated control cells for 24 (A) and 72 hrs (B).

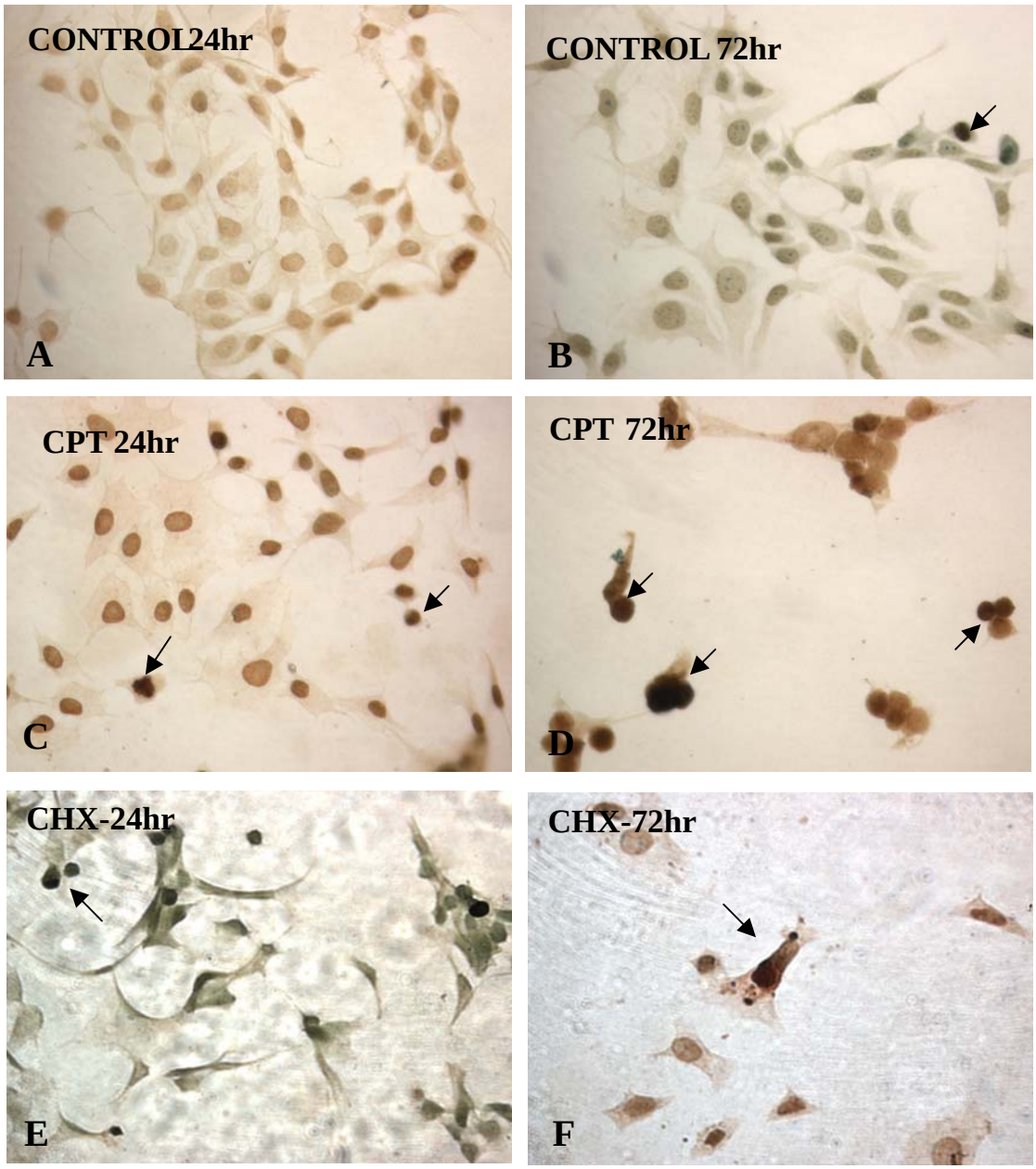
CONTROL24hr
A
CONTROL 72hr
B
CPT 24hr
C
CPT 72hr
D
CHX-24hr
E
CHX-72hr
F

**Figure 23**

**Figure 24. Nuclear fragmentation during cell death induced by CPT and CHX in cathepsin cells by Hoechst staining.**

Cells were prepared as described in Figure 19. Wild type and cathepsin B, D, and L null cells treated with CPT (50 μM) and CHX (100 μg/ml) were fixed with 4% paraformaldehyde and stained with Hoechst fluorescent dye. Hoechst staining demonstrated fragmented nuclei in dying cells. The frequency of fragmented nuclei was determined at different times (8, 24, and 72 hrs) by counting at 400X. The error bars represent standard deviation obtained from at least 3 independent experiments.

A. Nuclear fragmentation during cell death induced by CPT in wild type and cathepsin B -/-, cathepsin D -/- and cathepsin L -/- cells showed that there was an inhibition of nuclear fragmentation in cathepsin B, D and L -/- cells.

B. Nuclear fragmentation during cell death induced by CHX in wild type and cathepsin B -/-, cathepsin D -/- and cathepsin L -/- cells showed that there was a inhibition of nuclear fragmentation in cathepsin B, D and L -/- cells.

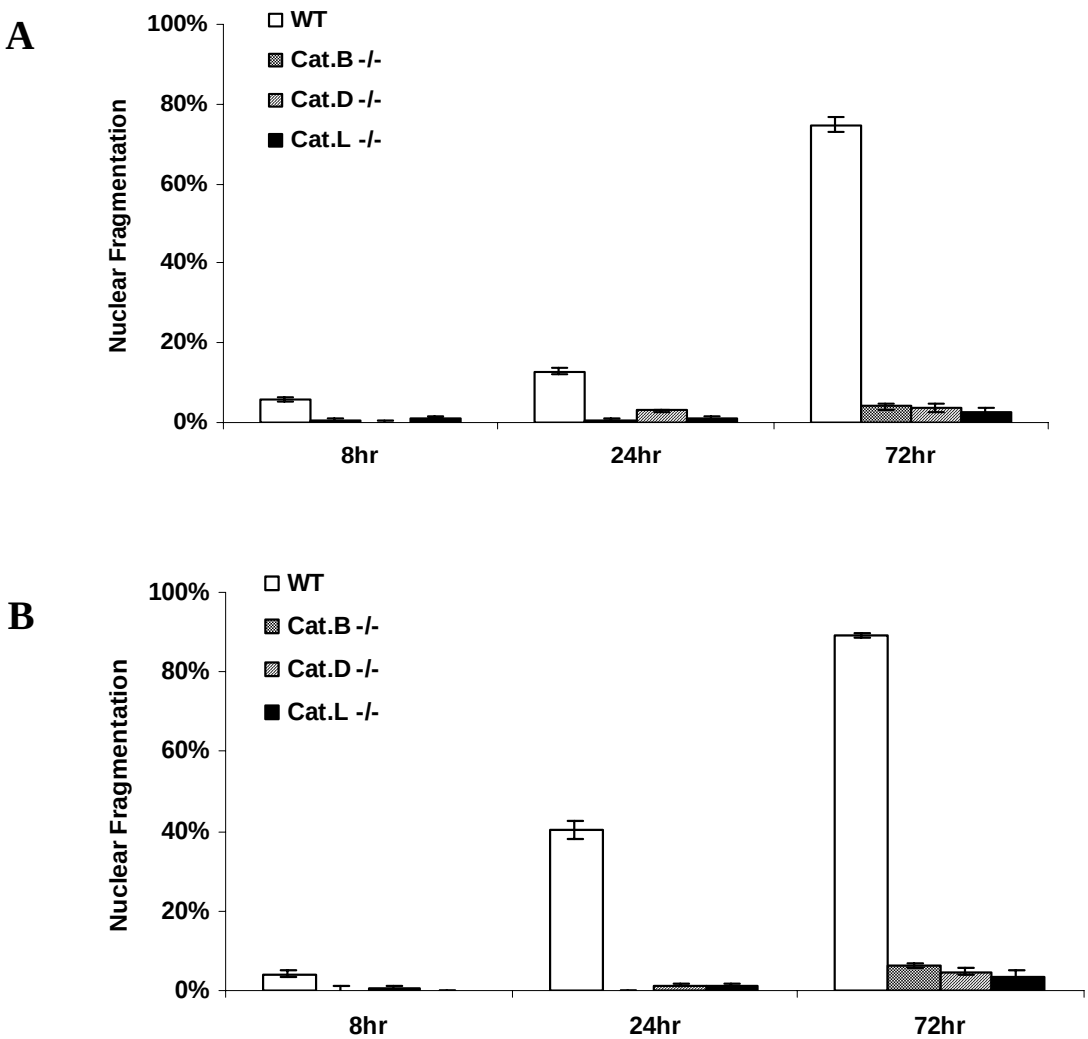
A
WT
Cat.B -/-
Cat.D -/-
Cat.L -/-
Nuclear Fragmentation
100%
80%
60%
40%
20%
0%
8hr
24hr
72hr
B
WT
Cat.B -/-
Cat.D -/-
Cat.L -/-
Nuclear Fragmentation
100%
80%
60%
40%
20%
0%
8hr
24hr
72hr

**Figure 24**

**Figure 25. Caspase-3 activation in wild type and cathepsin B -/-, cathepsin D -/-, and cathepsin L -/- cells.**

A, B, C, & D. Cells were prepared as described in Figure 19. Wild type and cathepsin B, D, and L -/- cells were incubated with different cell death inducers, including CPT (50 μM) and CHX (100 μg/ml) for 24, and 72 hrs. Proteins samples were isolated from cells with RIPA buffer. Western blot analysis of equal amount of protein from cells using anti-active caspase-3 antibody showed no induction of the active caspase-3 in wild type cells (A); cathepsin B -/- cells (B); cathepsin D -/- cells (C); cathepsin L -/- cells (D). 15% acrylamide gel was used.

E. DNA was extracted from wild type and cathepsin B, D, or L -/- cells and the caspase-3 genes were amplified by PCR as described in Materials and Methods. The products were separated on 2% agarose gel, showing no caspase-3 genes in cathepsin B, D, or L -/- cells. 1: cathepsin B -/- cells; 2: cathepsin D -/- cells; 3: cathepsin L -/- cells; 4: WT cells; 5: C8 cells (caspase-3 positive control cells).

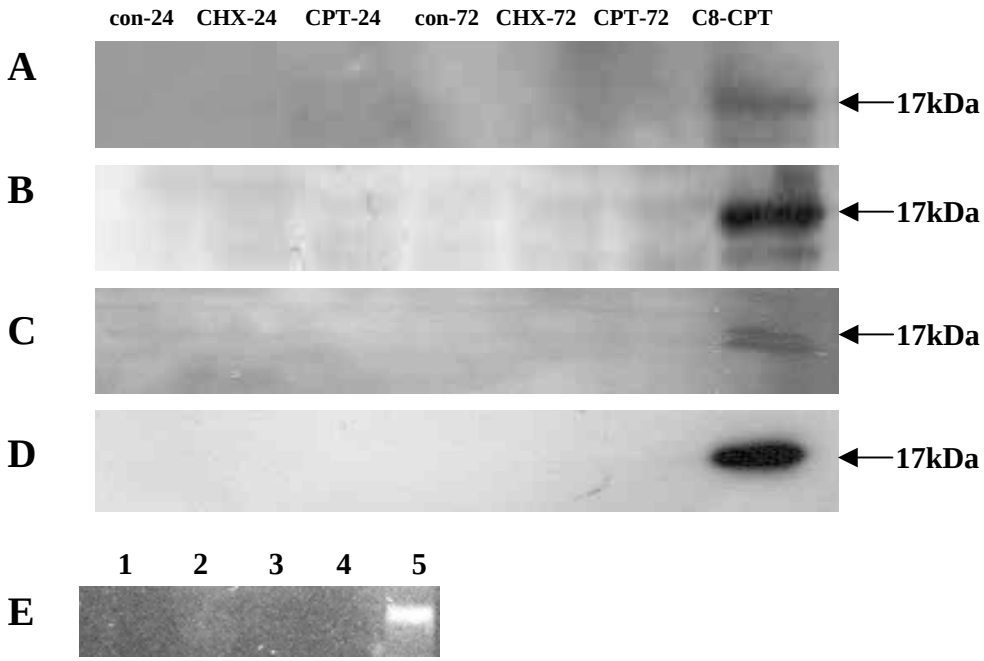
con-24
CHX-24
CPT-24
con-72
CHX-72
CPT-72
C8-CPT
A
17kDa
B
17kDa
C
17kDa
D
17kDa
1
2
3
4
5
E

**Figure 25**

**Figure 26. PARP cleavage and caspase-7 activation in wild type and cathepsin B -/-, cathepsin D -/-, and cathepsin L -/- cells.**

Cells were prepared as described in Figure 19. Wild type and cathepsin B, D, and L -/- cells were incubated with different cell death inducers, including CPT (50 μM) and CHX (100 μg/ml) for 72 hrs. Proteins were isolated from cells with RIPA buffer.

A & D. Western blot analysis of equal amounts of cell lysates from cells using anti-active caspase-7 antibody exhibited the activation of caspase-7 to form a 19kDa fragment in wild type cells; cathepsin B -/- cells; cathepsin D -/- cells; cathepsin L -/- cells. 15% acrylamide gel was used.

B & E. Western blot analysis of equal amount of cell lysates from cells using PARP antibody to exhibited the cleavage of PARP to form a 85kDa fragment in wild type cells; cathepsin B -/- cells; cathepsin D -/- cells; cathepsin L -/- cells. 8% acrylamide gel was used.

C & F. Western blot analysis of equal amount of cell lysates from cells using β -tubulin antibody demonstrated the equivalent loading of protein samples.

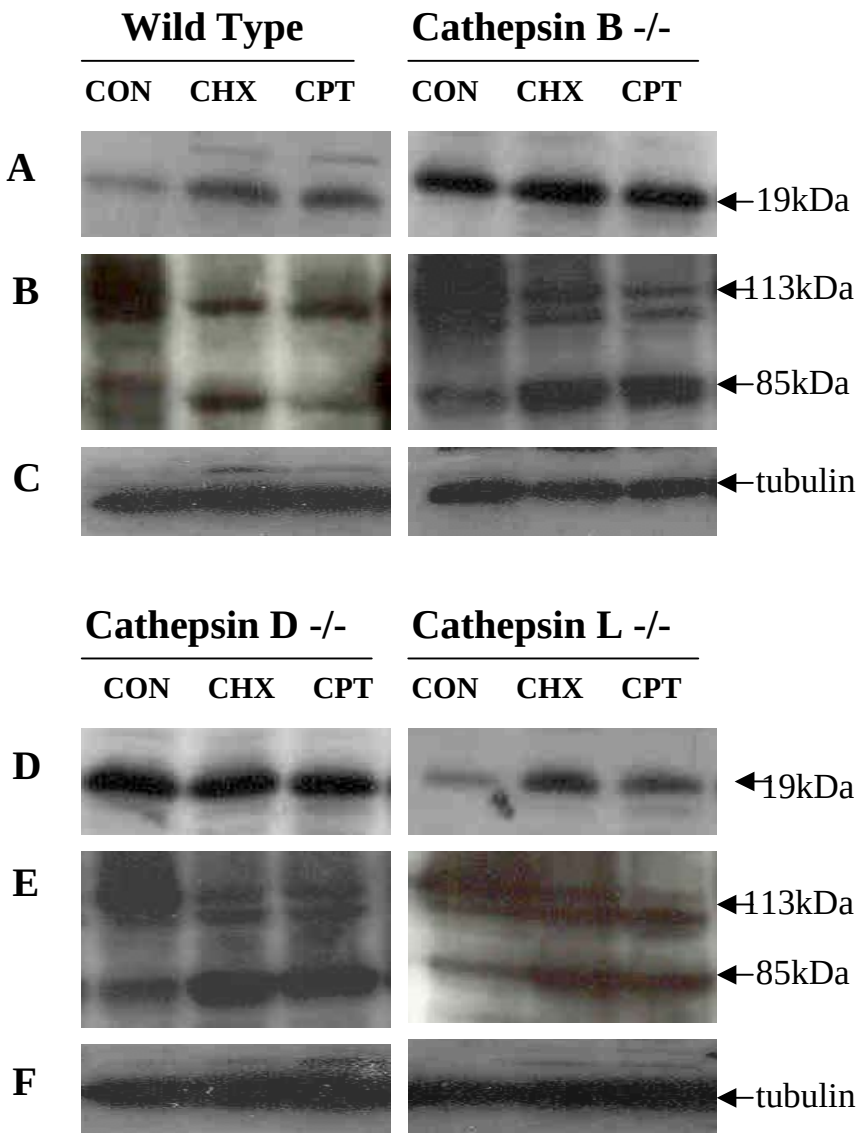
Wild Type
Cathepsin B -/-
CON CHX CPT
CON CHX CPT
A
B
C
19kDa
113kDa
85kDa
tubulin
Cathepsin D -/-
Cathepsin L -/-
CON CHX CPT
CON CHX CPT
D
E
F
19kDa
113kDa
85kDa
tubulin

**Figure 26**

**Figure 27. Morphological analysis of cell death in wild type cells by electron microscopy.**

Cells were cultured and treated as described in Figure 19. Wild type cells treated with CHX (100 μg/ml) and CPT (50 μM) were collected at 72 hrs and fixed for electron microscopy.

A. Untreated control wild type cells were elongated and displayed large normal nucleus.

B. Wild type cells treated with CPT showed cytoplasmic shrinkage, fragmentation and condensation of the chromatin, and increased electron density.

C. Wild type cells treated with CHX showed fragmentation and condensation of the chromatin, disintegrated outer membrane and the cytoplasm is not electron dense.

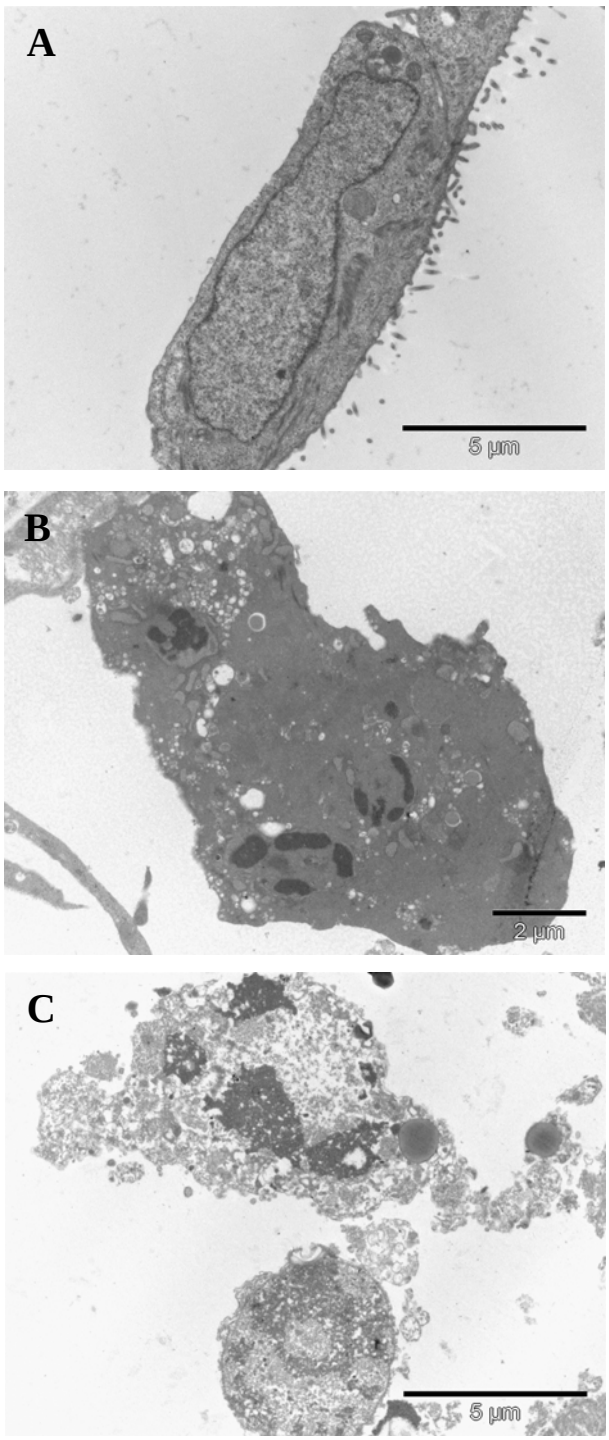


**Figure 27**

**Figure 28. Morphological analysis of cell death in cathepsin B -/- cells by electron microscopy.**

Cells were cultured and treated as described in Figure 19. Cathepsin B -/- treated with CHX (100 μg/ml) and CPT (50 μM) at 72 hrs were collected and fixed for electron microscopy. Untreated control cathepsin B -/- cells (A) were elongated and displayed large normal nuclei. Cathepsin B -/- cells treated with CPT (B) and CHX (C) showed nuclear condensation and fragmentation (B), chromatin redistribution without signs of either condensation or nuclear fragmentation (C), and an extensive presence of autophagic vacuoles of different sizes in the cell cytoplasm, often containing cell organelles.

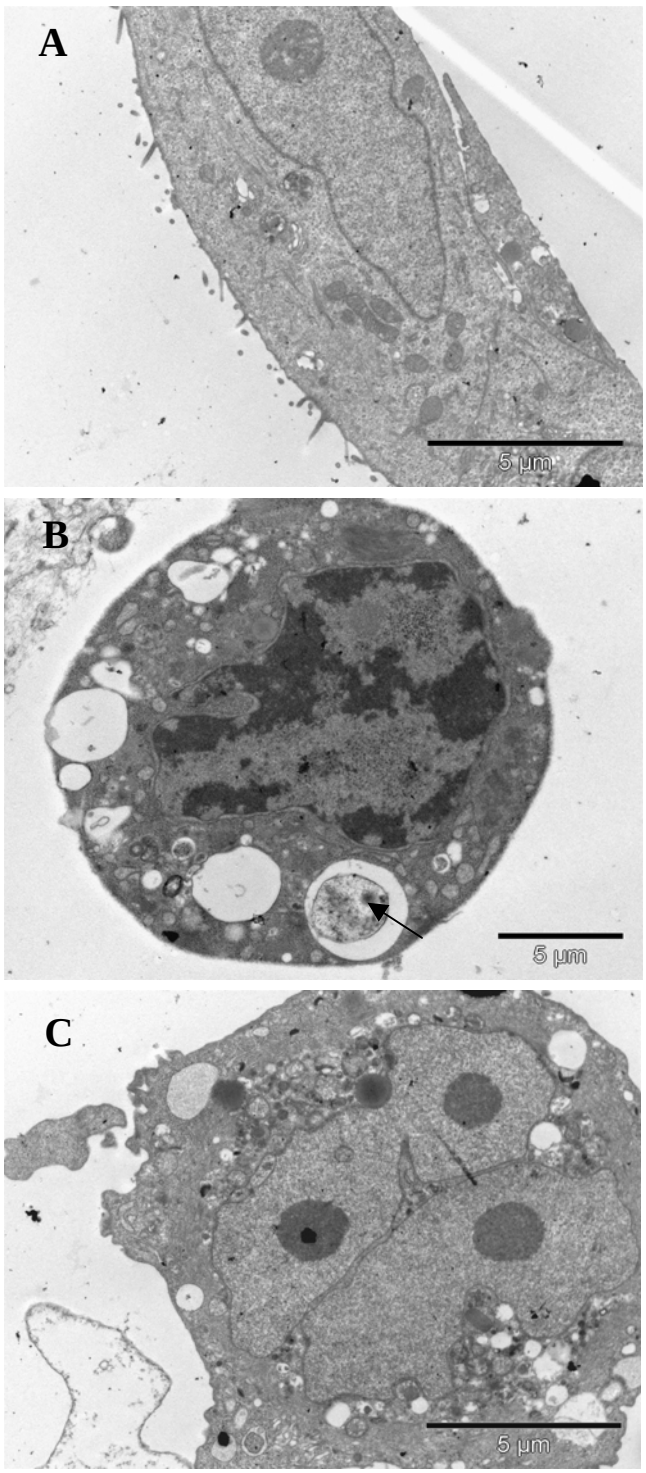


**Figure 28**

**Figure 29. Morphological analysis of cell death in cathepsin D -/- cells by electron microscopy.**

Cells were cultured and treated as described in Figure 19. Cathepsin D -/- treated with CHX (100 μg/ml) and CPT (50 μM) at 72 hrs were collected and fixed for electron microscopy. Untreated control cathepsin D -/- cells (A) were elongated and displayed large normal nuclei. Cathepsin D -/- cells treated with CPT (B) and CHX (C) showed nuclear condensation and fragmentation (B), chromatin redistribution without signs of either condensation or nuclear fragmentation (C), and an extensive presence of autophagic vacuoles of different size in the cell cytoplasm, often containing cell organelles.

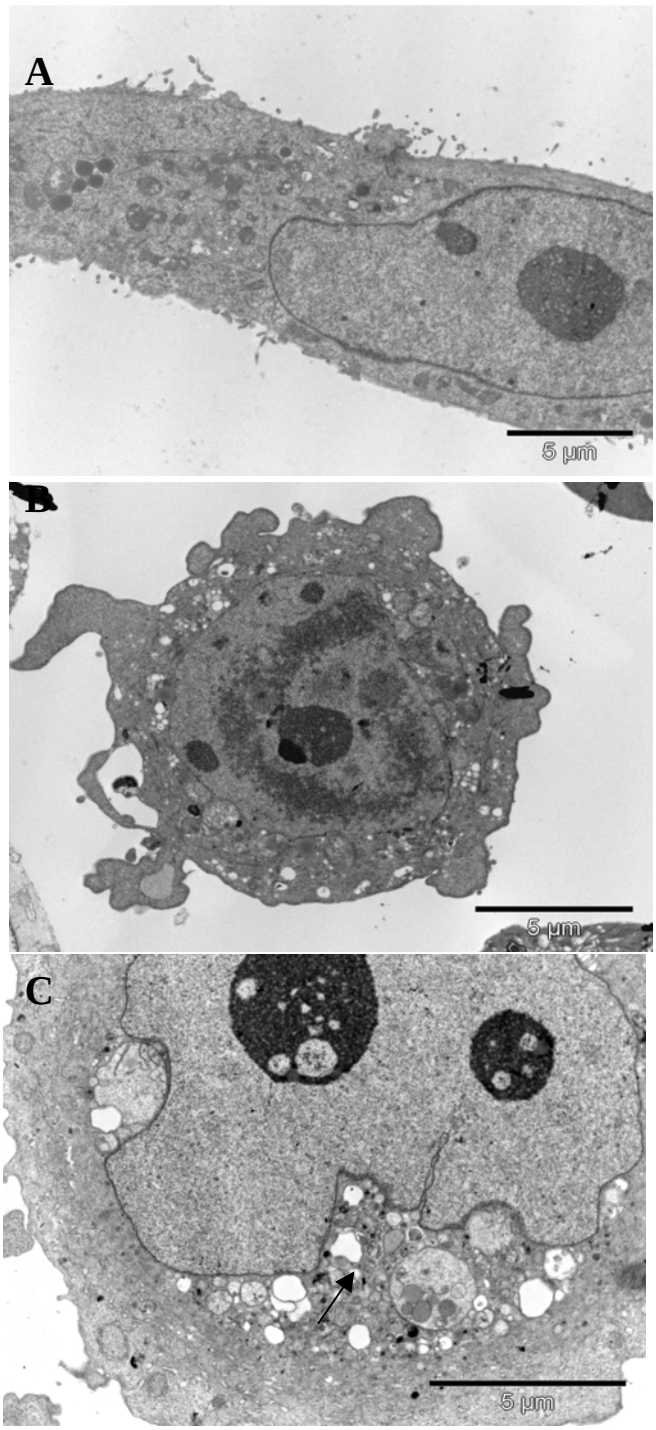


**Figure 29**

**Figure 30. Morphological analysis of cell death in cathepsin L -/- cells by electron microscopy.**

Cells were cultured and treated as described in Figure 19. Cathepsin L -/- treated with CHX (100 μg/ml) and CPT (50 μM) at 72hrs were collected and fixed for electron microscopy. Untreated control cathepsin L -/- cells (A) were elongated and displayed large normal nuclei. Cathepsin L -/- cells treated with CPT (B) and CHX (C) showed nuclear condensation and fragmentation (B), chromatin redistribution without signs of either condensation or nuclear fragmentation (C), and an extensive presence of autophagic vacuoles of different size in the cell cytoplasm.

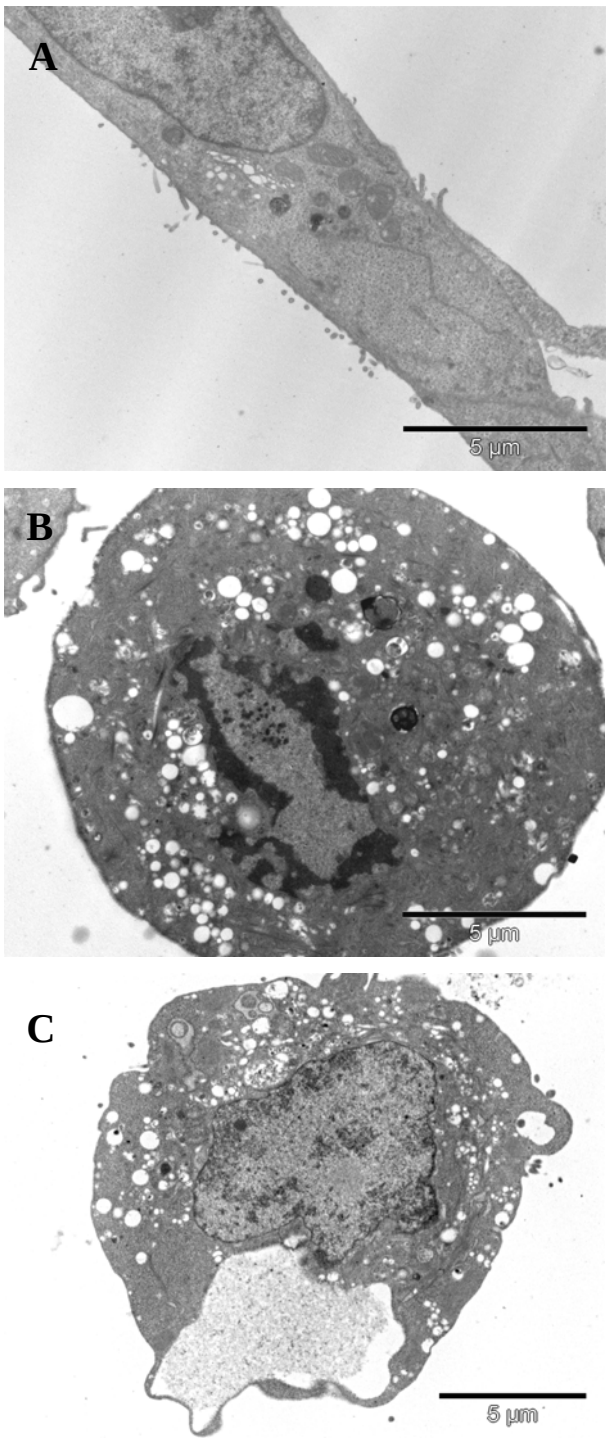


**Figure 30**

**Figure 31. Morphological analysis of cell death in cathepsin L -/- cells by electron microscopy.**

Cells were cultured and treated as described in Figure 19. Cathepsin L -/- treated with CHX (100 μg/ml) and CPT (50 μM) at 72 hrs were collected and fixed for electron microscopy. Untreated control cathepsin L -/- cells (A) were elongated and displayed large normal nuclei. Cathepsin L -/- cells treated with CPT (B) and CHX (C) showed nuclear condensation and fragmentation. In addition to the increase of vacuolization, an intact cell was seen in another cell.

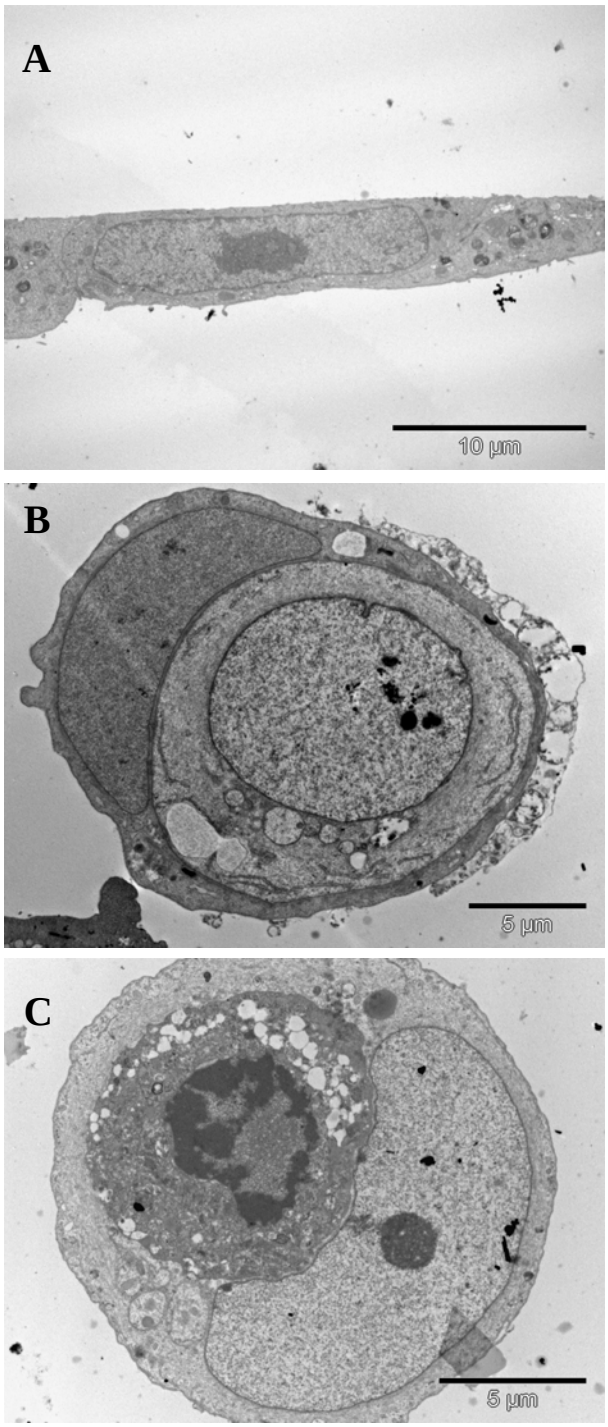


**Figure 31**

**Figure 32. Cannibalism induced by CHX in wild type, cathepsin B, D, or L -/- cells, and MCF-7 cells.**

Cells were stained by fluorescent CellTracker green or red. Cathepsin B, D, or L cells were exposed to 100 μg/ml CHX for 72 hrs, and WT and MCF-7 cells were exposed to CHX (100 μg/ml) for 48 hrs. A. Cells were incubated together to set up different groups: group #1: co-culture untreated control cells stained red with untreated control cells stained green; group #2: co-culture untreated control cells stained red with CHX-treated cells stained green; group #3: co-culture CHX-treated cells stained red with CHX-treated cells stained green. They were fixed in 4% paraformaldehyde and cell cannibalism was determined by confocal microscopy. B. Cannibalized cells were shown: red cell in green cell (a); green cell in green cell (b); green cell in red cell (b); red cell in red cell (c).

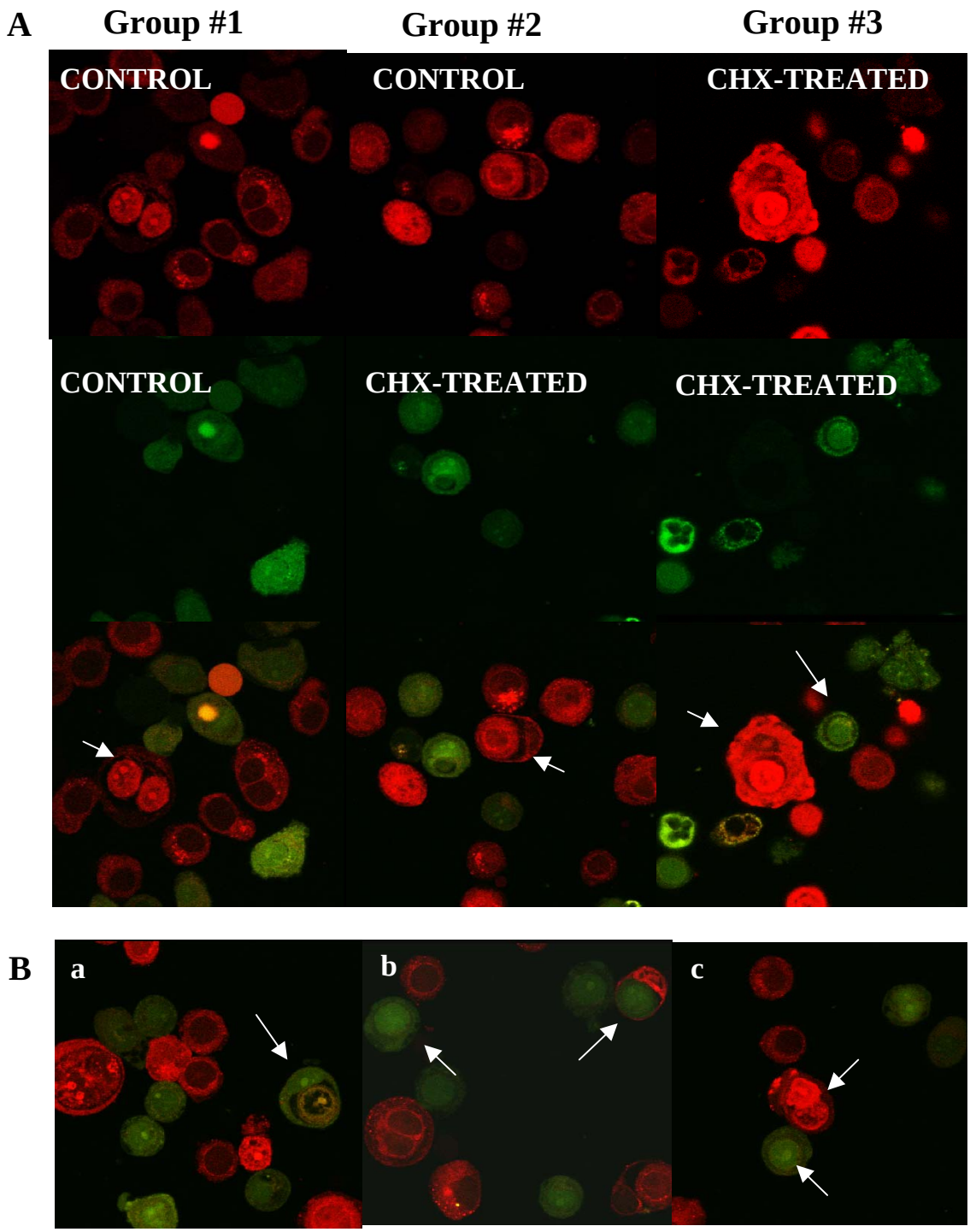
A
Group #1
Group #2
Group #3
CONTROL
CONTROL
CHX-TREATED
CONTROL
CHX-TREATED
CHX-TREATED
B
a
b
c

**Figure 32**

**Figure 33. Cannibalism induced by CHX in wild type, cathepsin B, D, or L -/- cells, and MCF-7 cells.**

Cells were prepared as described in Figure 32. Cell cannibalism was determined by confocal microscope and the percentage of cannibalized cells was determined. In group #3 of cathepsin D -/- and MCF-7 cells, and group # 2 and #3 of cathepsin L -/- cells, the amount of cannibalized cells was increased by comparing wild type cells. Group # 2 and #3 of Cathepsin D -/-, cathepsin L -/- and MCF-7 cells has higher amount of cannibalized cells compared with group #1 of these cells. The error bars represent the standard deviation from at least three individual experiments.

* p< 0.05 comparing to WT cells;

■ P< 0.05 comparing to group #1

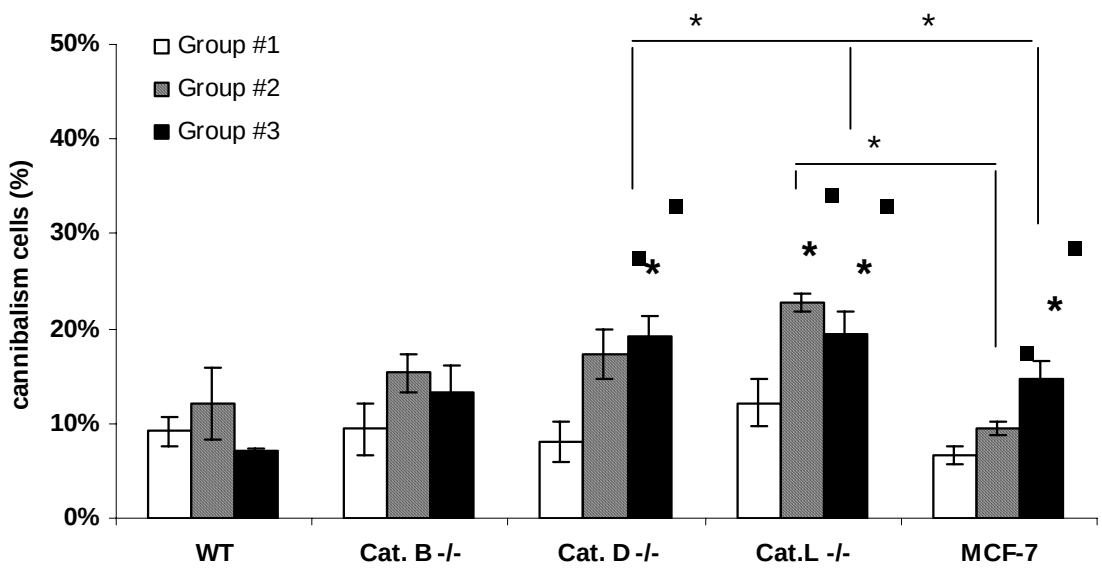


**Figure 33**

**Figure 34. Phagocytosis in wild type, cathepsin B, D, or L -/- cells, and MCF-7 cells.** Cells were exposed to CHX (100 μg/ml) or CPT (50 μM) for 48 hrs, and then cells were incubated with *Saccharomyces cerevisiae* zymosan A BioParticles for 2 hrs. After incubation, cells were fixed with 4% paraformaldehyde. The cells taking up particles were measured and the percentage of cells taking more particles (with higher intensity of fluorescence) is determined by FACS analysis. The error bars represent the standard deviation from at least three individual experiments. * $p < 0.05$ compared to WT cells (control to control, CHX to CHX, CPT to CPT).

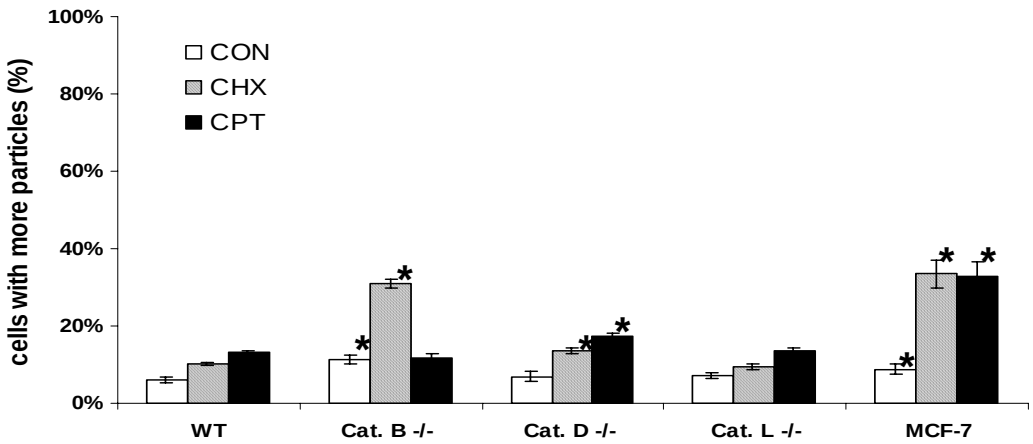
100%
80%
60%
40%
20%
0%
cells with more particles (%)
CON
CHX
CPT
*
WT
Cat. B -/-
Cat. D -/-
Cat. L -/-
MCF-7

**Figure 34**

**Figure 35. Cdk5 expression in wild type and cathepsin B -/-, cathepsin D -/-, and cathepsin L -/- cells.**

Wild type and cathepsin B, D, and L -/- cells were incubated with different cell death inducers, including CPT (50 μM) and CHX (100 μg/ml) for 8, 24, and 72 hr. Protein samples were isolated from cells with RIPA buffer. Western blot analysis of equal amount of cell lysates from cells using Cdk5 antibody showed an unchanged level of Cdk5 protein during cell death in wild type cells (A); cathepsin B -/- cells (B); cathespin D -/- cells (C); cathepsin L -/- cells (D). 12.5% acrylamide gel was used.

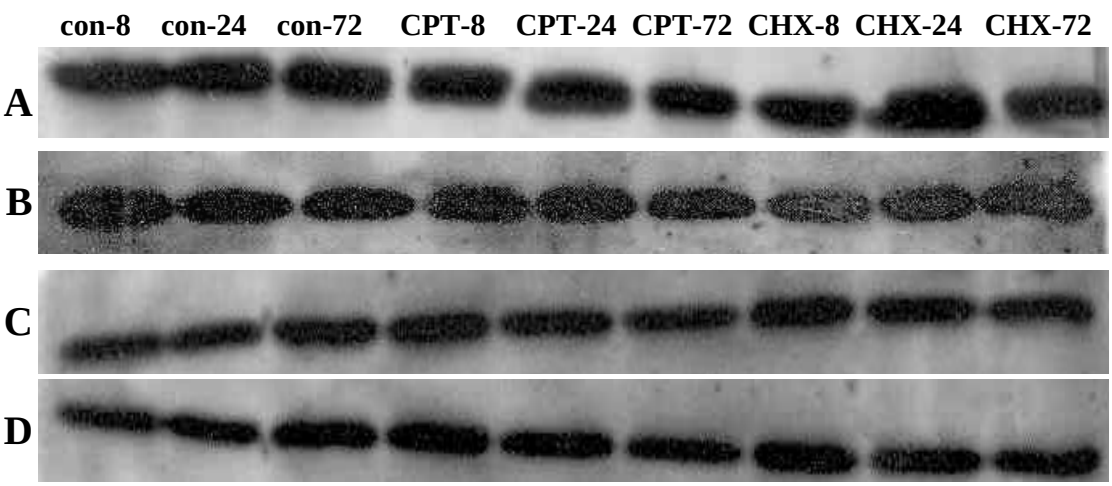


**Figure 35**

**Figure 36. Induction of p25 and activation of Cdk5 during cell death in both wild type and cathepsin B -/-, cathepsin D -/-, and cathepsin L -/- cells.**

Wild type and cathepsin B, D, and L -/- cells were incubated CPT (50 μM) and CHX (100 μg/ml) for 72 hrs. Protein samples were isolated from cells with RIPA buffer.

A & C. Western blot analysis of equal amount of cell lysates from cells using p35 antibody demonstrated an induction of p25 during cell death by CHX and CPT in wild type cells; by CPT in cathepsin B -/- cells; cathepsin D -/- cells; cathepsin L -/- cells. 12.5% acrylamide gel was used.

B & D. Histone H1 kinase activity of Cdk5 immunoprecipitates was induced during induced cell death in wild type cells; cathepsin B -/- cells; cathepsin D -/- cells; cathepsin L -/- cells. Following 72 hrs treatment with CHX and CPT, cells were collected and lysed with RIPA buffer. Kinase activity of Cdk5 immunoprecipitates was determined using histone H1 as i*n vitro* substrate. The histone H1 phosphorylated by Cdk5 was detected by western blot analysis using anti-phospho-histone H1 increased activity corresponding to cell death. 12.5% acrylamide gel was used.

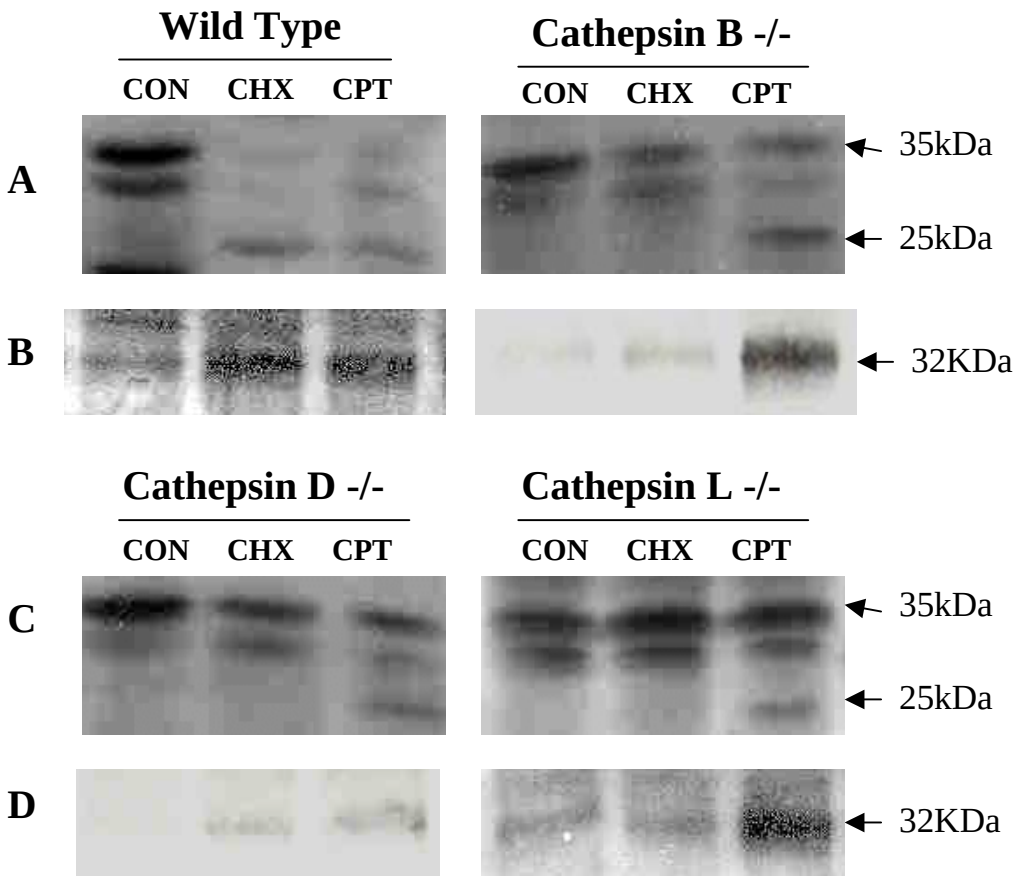
Wild Type
CON CHX CPT
Cathepsin B -/-
CON CHX CPT
A
35kDa
25kDa
B
32KDa
Cathepsin D -/-
CON CHX CPT
Cathepsin L -/-
CON CHX CPT
C
35kDa
25kDa
D
32KDa

**Figure 36**

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