CHAPTER 1

Introduction

CELL DEATH

Living cells are equipped with highly efficient death machinery that is readily activated when they reach their duly allotted life span or are deemed to be ineffectual, redundant, or damaged. Until 1971, the term "necrosis" was used for all types of cell death. When Kerr *et al.,* (1971) first observed a form of nonpathologic cell death in certain tissues, they termed it shrinkage necrosis. As shrinkage necrosis became implicated in the control of organ homeostasis, it was renamed as apoptosis (Kerr *et al.,* 1972). Over the last three decades, apoptotic cell death has been well characterized at both the genetic and biochemical levels.

1.1 Necrosis

Necrosis (from the Greek νεκρός, means "dead") is the premature death of cells and living tissue. Necrosis is caused by factors external to the cell or tissue, such as infection, toxins, or trauma. While apoptosis often provides beneficial effects to the organism, necrosis is almost always detrimental and can be fatal. Necrosis is a type of cell death caused by severe (non-physiological) physical and/or chemical insults and usually affecting groups of cells (Majno and Joris, 1995).

1.1.1 Morphology of Necrosis

The morphology of a necrotic cell is very distinct from that of a cell undergoing apoptosis, with ultrastructural changes occurring in both the cytoplasm and the nucleus. The main characteristic features are chromatin flocculation, swelling and degeneration of the entire cytoplasm and the mitochondrial matrix, blebbing of the plasma membrane, and eventual shedding of the cytoplasmic contents into the extracellular space (Figure 1.1) (Kerr *et al.,* 1971). Unlike in apoptosis, the chromatin is not packed into discrete membrane-bound particles, but it forms many unevenly textured and irregularly shaped clumps, a feature that is being used for differentiating between the two modes of cell death (Trump *et al.,* 1965). The mitochondria undergo inner membrane swelling, cristeolysis, and disintegration (Laiho *et al.,* 1971). Polyribosomes are dissociated and dispersed throughout the cytoplasm, giving the cytoplasmic matrix a dense and granular appearance. Dilation and fragmentation of

the cisterns of rough endoplasmic reticulum and Golgi apparatus are frequently observed (Trump *et al.*, 1965).

1.1.2 Biochemical features of necrosis include loss of regulation of ion hemostasis, the process is uncontrolled and passive and does not require energy. Severely damaged cells do not form membrane-bound vesicles (apoptotic bodies such as observed during apoptosis), and thus release their cellular contents. This normally results in inflammatory reactions with oedema and damage to surrounding cells. These effects of necrosis are exacerbated during neuronal necrosis because neurotransmitters that are released by dying cells can cause excitotoxic injury and cell death to their neighbours. Random DNA fragmentation occurs after lysis. However, recent data from several studies indicate that discriminating between apoptosis and necrosis based on DNA fragmentation pattern is questionable, because both modes of cell death can occur in the absence or presence of DNA fragmentation (Dong *et al.*, 1997; Rich *et al.*, 2000; Ueda and Shah, 2000). DNA fragmentation represents a point of no return from the path to cell death, because no more new cellular protein synthesis for cell survival can occur.

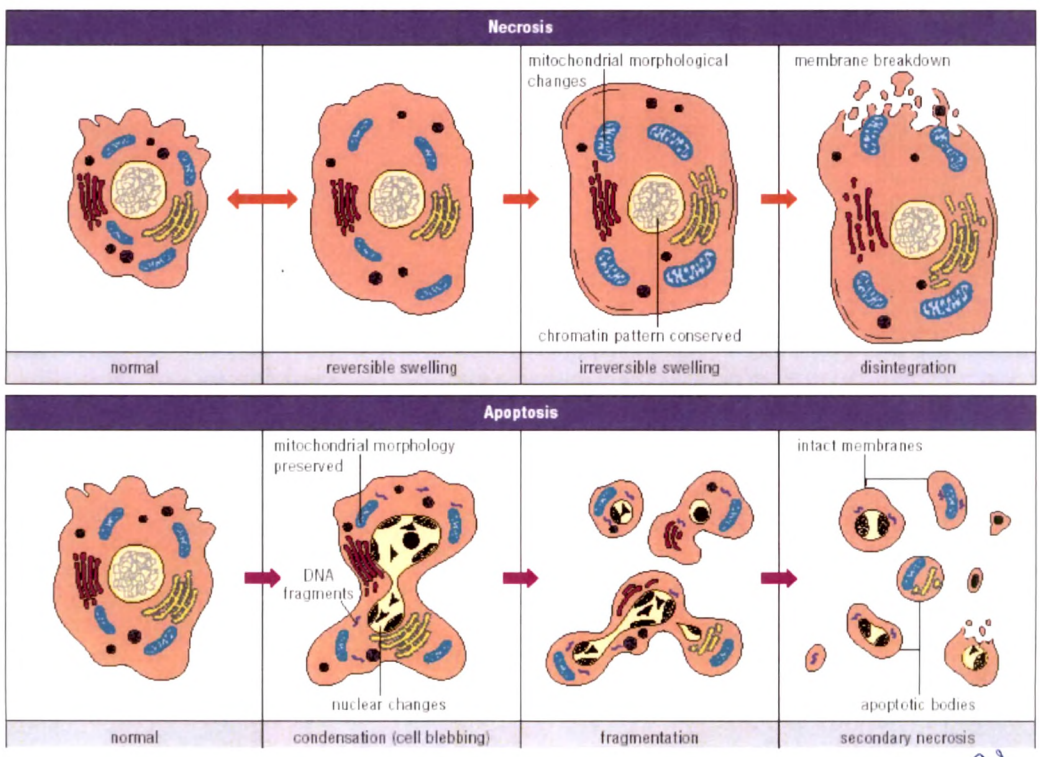
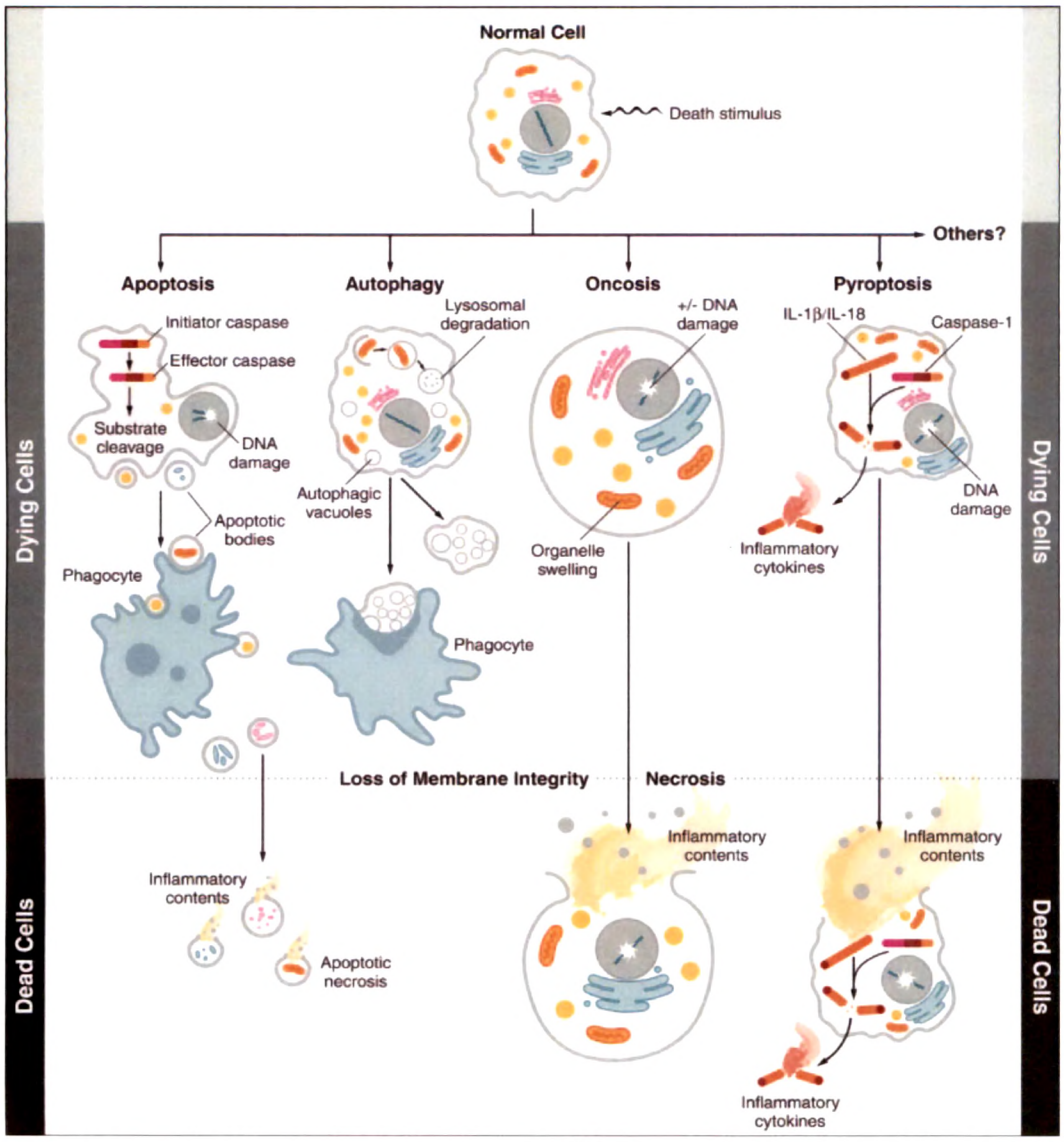


Figure 1.1 Schematic diagram of Necrosis and apoptosis



(Fink and Cookson, 2005)

Figure 1.2 Pathways leading to cell death. Healthy cells respond to death-inducing stimuli by initiating a variety of molecular pathways leading to cell death. Completion of the proper pathway is a critical cellular function to ensure that the appropriate outcome is ultimately achieved in a multicellular organism. Failure to die in response to particular stimuli can result in abortive embryogenesis and organ dysfunction and contributes to the initiation of cancer. Proinflammatory death is vital in triggering appropriate immune responses or, in the extreme, may cause tissue pathology and organ dysfunction. Therefore, pathway utilization can dramatically influence biological systems. Apoptosis is a pathway leading to cell death that features the activation of initiator caspases that activate effector caspases to cleave

cellular substrates. Apoptotic cells demonstrate cytoplasmic and nuclear condensation, DNA damage, formation of apoptotic bodies, maintenance of an intact plasma membrane, and exposure of surface molecules targeting intact cell corpses for phagocytosis. In the absence of phagocytosis, apoptotic bodies may proceed to lysis and secondary or apoptotic necrosis. Autophagy features degradation of cellular components within the intact dying cell in autophagic vacuoles. The morphological characteristics of autophagy include vacuolization, degradation of cytoplasmic contents, and slight chromatin condensation. Autophagic cells can also be taken up by phagocytosis. Oncosis is the prelethal pathway leading to cell death accompanied by cellular and organelle swelling and membrane breakdown, with the eventual release of inflammatory cellular contents. Pyroptosis is a pathway to cell death mediated by the activation of caspase-1, a protease that also activates the inflammatory cytokines, IL-1ß, and IL-18. This pathway is therefore inherently proinflammatory. Pyroptosis also features cell lysis and release of inflammatory cellular contents. Undoubtedly, other pathways exist that have not yet been described.

The process outlined above is also known as primary necrosis or oncosis (type 1 necrosis). The presence of necrotic cells does not necessarily imply that they did not die by apoptosis. The term secondary necrosis (type 2 necrosis) refers to a process in which late stage apoptotic cells that failed to be engulfed by phagocytes or neighbouring cells undergo necrosis. Secondary necrosis, thus, is a post-apoptotic event. This process is seen in cultured cells that are undergoing cell death by apoptosis *in vitro*, induced, for example, by the absence of survival factor signals or activation of death receptors by different lethal signals. These cells, in the absence of phagocytic cells that could engulf them, ultimately cease to be metabolically active, lose membrane integrity, and release their cytoplasmic contents into the culture medium. Secondary necrosis can also occur *in vivo*, for example in autoimmune disorders associated with impaired clearance of apoptotic cells. Secondary necrosis may be seen also during massive local apoptosis when phagocytes and neighbouring cells may be unable to cope with the load of apoptotic cells. Therefore, the process plays a critical role in inflammation, tissue remodeling, and immune response regulation (Aderem and Underhill, 1999; Silva *et al*, 2008).

Despite the idea that necrosis is an uncontrolled or default form of cell death, accumulating studies have suggested that this may not be true. Rather, it appears that necrotic cell death can be a regulated event that contributes to development and to the maintenance of organism homeostasis. Terms such as autolysis, oncosis (Majno and Joris, 1995), pyroptosis (Cookson and Brennan, 2001) and necrapoptosis (Lemasters, 1999) have been used to describe modes of nonapoptotic cell death that display aspects of programmed cellular suicide. Evidences are there that metazoan cells can initiate their own death by necrosis. The genetic components of this programmed cell necrosis involve (1) gene products that function in the dying cell to induce an irreversible bioenergetic compromise that results in cell death, and (2) gene products that are selectively released into the extracellular environment to trigger a host response. The evolutionary advantage conferred by necrosis is that it allows cells to actively recruit a defensive response to regions of multicellular organisms that have sustained damage or invasion. Programmed cell necrosis can be a consequence of extracellular signaling or can be initiated as a form of cellular suicide in response to intracellular perturbations.

1.1.3 Physiological roles and significance of necrosis

The core events of necrosis are bioenergetic failure and rapid loss of plasma membrane integrity. These can result from defined molecular events that occur in the dying cell, including increased mitochondrial ROS production, channel-mediated calcium uptake, activation of nonapoptotic proteases, and/or enzymatic destruction of cofactors required for ATP production. In addition, these necrotic mediators are often induced in the dying cell simultaneously and potentiate each other's ability to initiate the demise of the cell. There are several physiological, pathological, and pharmacological conditions where necrosis may play an important role. Cell suicide by necrosis appears to have evolved to allow multicellular organisms to have an early warning system to recognize and adapt to events that might compromise the integrity of the organism as a whole. As such programmed cell necrosis plays a role in a number of disease processes including vascular-occlusive disease, neurodegenerative diseases, infection, inflammatory diseases, exposures to toxins, and cancer (Majno and Joris 1995; Proskuryakov *et al.* 2002; Yuan *et al.* 2003).

1.1.4 Molecular mechanism of programmed cell necrosis

The fundamental feature that distinguishes most forms of necrosis from apoptosis is the rapid loss of cellular membrane potential. The inability to maintain these electrochemical potential results in cytoplasmic swelling, rupture of the plasma membrane, and cytolysis. This loss of membrane potential may be a consequence of cellular energy depletion, damage to membrane lipids, and/or loss of function of homeostatic ion pumps/channels. These defects can synergize in the induction of necrotic cell death. For example, defective ATP production and/or excessive ATP consumption can lead to a reduction in function of the ATP-dependent ion pumps on the plasma membrane. Perturbation of intracellular ion homeostasis can result in mitochondrial dysfunction and diminished ATP production.

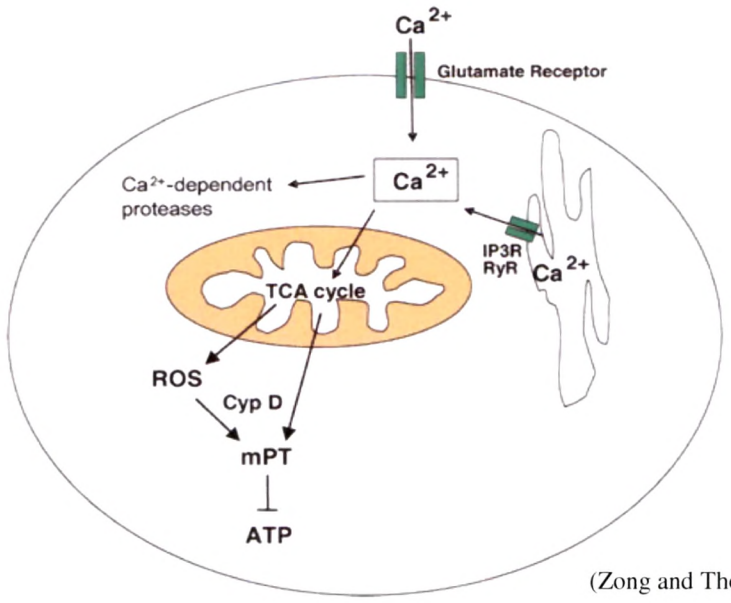
1.1.4.1 Calcium as a mediator of necrotic cell death

Intracellular Ca^{2+} is an important signaling molecule for numerous cell responses including necrosis. In certain pathological conditions, extracellular ligands can induce Ca^{2+}-dependent necrosis. One good example is the excitotoxic neuronal cell death induced by excitatory amino acids such as N-methyl-d-aspartate (NMDA) class of glutamate receptor.

In viable cells the plasma membrane and intracellular membranes are virtually impermeable to Ca2+. Under physiological conditions the Ca2+ concentration is ~1.2 mM extracellularly, and ~0.1 μM in the cytosol. Most of intracellular Ca2+ is stored in the ER. When the ER Ca2+ is released into the cytosol, or the extracellular Ca2+ crosses the plasma membrane, cell death can be initiated due to the activation of Ca2+ dependent proteases and/or mitochondrial Ca2+ overload.

Ca^{2+} mediated necrosis has been the best characterized form of programmed necrosis. In *Caenorhabditis elegans*, gain-of-function (hyperactive) mutants of the DEG/ENaC family members MEC-4 and MEC-10 [MEC-4(d) and MEC-10(d)] induces necrosis of touch neurons (Driscoll and Chalfie 1991; Huang and Chalfie 1994). MEC-4(d)-induced neuronal cell death is independent of C. elegans apoptosis regulators,

although genes required to remove the necrotic corpse are the same as those for the apoptotic corpse (Chung et al. 2000). Expression of MEC-4(d) induces Ca^{2+} influx and activates Ca^{2+} induced ER Ca^{2+} release that leads to necrosis (Xu *et al.*, 2001; Bianchi *et al.*, 2004) (Figure 1.3).



(Zong and Thompson, 2006)

Figure 1.3 Calcium-mediated programmed necrosis. Intracellular calcium increases in response to the activation of ionotrophic glutamate receptors or through other calcium channels on the plasma membrane or the ER membrane. An intracellular calcium spike induces the activation of Ca^{2+}-dependent proteases and stimulates mitochondrial TCA cycle activity and ROS production. If sustained, the resulting ROS leads to mPT (Mitochondrial permibility transition) that is dependent on CypD (cyclophilin D). mPT then leads to the loss of ATP production and necrosis.

Like many other insults, increased cytosolic Ca^{2+} can initiate either apoptosis or necrosis. The outcome of cell death is probably determined by the concentration of cytoplasmic Ca^{2+}; low to moderate Ca^{2+} (200–400 nM) triggers apoptosis, higher concentration of Ca^{2+} (>1 μM) is associated with necrosis (McConkey and Orrenius, 1996). This may explain why Ca^{2+} released from the ER is mostly apoptotic, whereas Ca^{2+} influx through the plasma membrane is associated with necrosis. The mitochondrial metabolic status may also affect the sensitivity of mitochondria to Ca^{2+} poisoning and contribute to the determination of the death mode (Ankarcrona *et al.*, 1995).

1.1.4.2 ROS-initiated necrosis

Cells in an aerobic environment are constantly generating ROS. Physiological levels of ROS can regulate transcription, serve as signaling molecules, and defend against pathogen infection. Excessive production of ROS leads to oxidative stress, damage of intracellular molecules and organelles, and ultimately necrosis.

ROS include a number of molecules derived from oxygen. They include molecules with an unpaired electron, often termed free radicals. Some major species include superoxide ($O2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$), nitric oxide (NO) and peroxynitrite. They are generated primarily in the electron-rich environment of the mitochondrial inner membrane, or through endogenous enzymes such as flavoenzymes, xanthine oxidase, NADPH-oxidase, glucose oxidase, nitric oxide synthase, or transition metals (Andreyev *et al.*, 2005). ROS are neutralized by endogenous enzymes such as superoxide dismutase (SOD), glutathione peroxidase, catalase, thioredoxin reductase, or by reacting with a free radical scavenger such as the endogenous glutathione and exogenous antioxidants (Nordberg and Arner, 2001).

Mitochondria are a major source of ROS that can initiate necrosis. Excess mitochondrial ROS can damage nuclear DNA by causing cleavage of DNA strands, DNA–protein cross-linking, and oxidation of purines (Marnett, 2000). This may lead to DNA-damage response, including activation of p53 and PARP. While activation of p53 may cause apoptosis and cell cycle arrest, hyperactivation of PARP leads to necrosis. Inhibition of PARP activity with inhibitors or gene knockdown blocks necrosis induced by H_2O_2 (Yu *et al.*, 2002). PARP-deficient animals are resistant to ischemia–reperfusion injury (Endres *et al.*, 1997). ROS also modify lipids, as the double bonds in polyunsaturated fatty acids are excellent targets for ROS attacks. Lipid oxidation can lead to the loss of integrity of both the plasma membrane and intracellular membranes such as that of lysosomes and the ER, leading to an intracellular leak of proteases or an influx of Ca^{2+} and resulting in necrosis. Another target of ROS is amino acid residues with sulfur or proteins with sulfhydrl links. ROS can attack the disulfide bond, or break up the sulfhydrl links, thereby changing the function of the modified proteins. ROS-mediated protein modification contributes to

necrosis through modifying Ca^{2+} channels. Thus emptying of intracellular calcium stores and/or alteration in intracellular calcium levels can modulate death in almost all cell types. These calcium fluxes are determined by the activity of membrane channels normally under tight control. The channels may be ligand activated or voltage dependent as well as being under the control of affector molecules such as calmodulin. It has become increasingly apparent that many calcium channels are affected by reactive oxygen or reactive nitrogen species; ROS/RNS. This may be part of the normal signaling pathways in the cell or by the action of exogenously generated ROS or RNS often by toxins. Both the ER and plasma membrane Ca^{2+} channels have been shown to be affected by ROS, and the resulting influx of Ca^{2+} can trigger necrosis (Waring, 2005).

1.1.4.3 Proteases involved in necrosis

A number of proteases have been implicated in necrosis. Calpains and cathepsins are the major proteases involved in necrosis. Genetic evidence for the involvement of these proteases in necrosis came from studies in *C. elegans*. RNAi silencing of CLP-1 and TRA-3 (homologs of mammalian calpain proteases) and ASP-3 and ASP-4 (homologs of mammalian cathepsin proteases) reduced the neuronal necrosis (Syntichaki *et al.*, 2002).

1.1.4.3.1 Calpains

Calpains are a family of Ca^{2+}-dependent cysteine proteases. Calpains reside in the cytosol in inactive form. In response to increased levels of cytosolic Ca^{2+}, calpains translocate to the intracellular membranes and are activated by autocatalytic hydrolysis. A number of calpain substrates have been identified including cytoskeletal proteins, membrane proteins, adhesion molecules, ion transporters, kinases, phosphatases, and phospholipases (Rami, 2003).

Calpain-mediated cleavage of the Na^{+}/Ca^{2+} exchanger in the plasma membrane results in the sustained secondary intracellular Ca^{2+} overload and subsequent necrotic cell death (Bano *et al.*, 2005). Calpain may also contribute to the activation of cathepsins

by causing lysosomal membrane permeability (LMP), which can lead to release of lysosomal enzymes and necrotic cell death (Yamashima, 2004).

1.1.4.3.2 Lysosomal enzymes

A critical point to determine the cell death mode is at the regulation of the lysosomal membrane permeabilization (LMP). One of the molecules that have been shown to regulate LMP is sphingosine, one of the membrane sphingolipid metabolites.

In response to cell death stimuli, the lysosomal enzyme sphingomyelinase is activated and converts sphingomyelin, the major sphingolipid, into ceramide. Ceramide can be further converted into sphingosine by ceramidase. Both ceramide and sphingosine have been shown to be important signaling molecules that induce apoptosis (Cuvillier, 2002). In addition, sphingosine, which is also a detergent, has been shown to cause LMP and cell death in a dose-dependent manner. Sphingosine induces partial lysosomal rupture and apoptosis at low-to-moderate concentrations (<20 μM), and extensive lysosomal rupture and necrosis at high concentration (>20 μM) (Kagedal *et al.*, 2001). Apoptosis, necrosis, and autophagic response can all be initiated from lysosomes. This places lysosomes as a critical control point for cell-fate determination; to which direction the cells go may be dependent on the magnitude of the LMP.

1.2 Apoptotic cell death or caspase-dependent PCD

The word Apoptosis is derived from a word of ancient Greek origin: απόπότσισ (falling off). This literally means falling off or falling away as in leaves from a tree in autumn which incidentally also involves apoptosis. In all living organisms there is a harmonious balance between the synthesis of new cells and the destruction of existing cells under normal conditions. This balance is particularly vital in maintaining both proper function and structure within the organism. The complex events that encompass these processes take place in response to specific and controlled signals.

Apoptosis is a physiological process that occurs via an extrinsic or intrinsic (mitochondrial) pathway. Both of these pathways involve caspase cascade, which eventuates in the death of the cell. This process is an essential part of normal physiological function and as a result approximately between 50 billion and 70 billion cells die each day due to apoptosis in the average human adult i.e. blood cells and those in epithelia lining organs such as the intestine. In a year, this amounts to the proliferation and subsequent destruction of a mass of cells equal to an individual's body weight. Apoptosis has always been recognized to be a pathway of highly orchestrated signalling events and is still often referred to as synonymous to programmed cell death (PCD). Apoptosis is highly coordinated and is generally thought to be mediated by active intrinsic mechanisms, although extrinsic factors can contribute (Bellamy *et al.,* 1995; Chalmers-Redman *et al.,* 1997; Schwarz *et al.,* 2000). Apoptosis is genetically controlled and is defined by cytoplasmic and nuclear shrinkage, chromatin margination and fragmentation, and breakdown of the cell into multiple spherical bodies that retain membrane integrity (Buja *et al.,* 1993; Kerr *et al.,* 1972; Majno and Joris, 1995; Wyllie *et al.,* 1980).

1.2.1. Physiological roles and significance of apoptosis

Apoptosis occurs as an integral component of normal development of the embryo, maturation and cell turnover. A frequent example is the development of a tadpole into a frog. Its a homeostatic process which is carried out by cells of living organisms as a means of eliminating unwanted cells and to maintain cell population in tissue,and also the cells that have been exposed to injurious or infectious agents rendering them damaged and potentially dangerous to the rest of the organism.

This defense can occur as a response to viral or bacterial infection, damage to DNA, for example by mutagenic agents, genetic disorders, autoimmune disease and various other injurious agents. In the immune system cells infected by a virus may recognise the infectious agent triggering the process of apoptosis. Apoptosis acts to destroy the cell to prevent the virus from replicating and infecting further cells of the organism. On the contrary, too little apoptosis is of particular significance in the formation of

cancer, essentially due to mutations in cancer cells that prevent them from undergoing apoptosis. Apoptosis has also been observed in plants, particularly higher plants. The xylem in plants consists of spaces that result from the death of cells that previously occupied those spaces, comparable to the digitization of the hand during embryological development (Elmore, 2007).

1.2.2 Morphology of apoptosis

Light and electron microscopy have identified the various morphological changes that occur during apoptosis (Hacker, 2000). During the early process of apoptosis, cell shrinkage and pyknosis are visible by light microscopy (Kerr *et al.,* 1972). With cell shrinkage, the cells are smaller in size, the cytoplasm is dense and the organelles are more tightly packed. Pyknosis is the result of chromatin condensation and this is the most characteristic feature of apoptosis. Extensive plasma membrane blebbing occurs followed by karyorrhexis and separation of cell fragments into apoptotic bodies during a process called "budding." Apoptotic bodies consist of cytoplasm with tightly packed organelles with or without a nuclear fragment. The organelle integrity is still maintained and all of this is enclosed within an intact plasma membrane. These bodies are subsequently phagocytosed by macrophages, parenchymal cells, or neoplastic cells and degraded within phagolysosomes. Macrophages that engulf and digest apoptotic cells are called "tingible body macrophages" and are frequently found within the reactive germinal centers of lymphoid follicles or occasionally within the thymic cortex. The tingible bodies are the bits of nuclear debris from the apoptotic cells. There is essentially no inflammatory reaction associated with the process of apoptosis nor with the removal of apoptotic cells because: (1) apoptotic cells do not release their cellular constituents into the surrounding interstitial tissue; (2) they are quickly phagocytosed by surrounding cells thus likely preventing secondary necrosis; and, (3) the engulfing cells do not produce anti-inflammatory cytokines (Savill and Fadok, 2000; Kurosaka *et al.,* 2003). *In vitro*, in the absence of phagocytosis, apoptotic bodies ultimately swell and lyse, and this terminal process of cell death has been termed "secondary necrosis". Secondary necrosis may occur *in vivo* in autoimmune disorders associated with impaired clearance of apoptotic cells (Wu *et al.,* 2001).

1.2.3 Biochemical features of apoptosis

Apoptotic cells exhibit several biochemical modifications such as protein cleavage, protein cross-linking, DNA breakdown, and phagocytic recognition (Hengartner, 2000). Caspases are widely expressed in an inactive proenzyme form in most cells and once activated can often activate other procaspases, allowing initiation of a protease cascade. Some procaspases can also aggregate and autoactivate. This proteolytic cascade, in which one caspase can activate other caspases, amplifies the apoptotic signaling pathway and thus leads to rapid cell death.

Extensive protein cross-linking is another characteristic of apoptotic cells and is achieved through the expression and activation of tissue transglutaminase (Nemes *et al.,* 1996). DNA breakdown by Ca^{2+}-and Mg^{2+}-dependent endonucleases or CAD (caspase activated DNase) also occurs, resulting in DNA fragments of 180 to 200 base pairs (Bortner *et al.,* 1995). A characteristic "DNA ladder" can be visualized by agarose gel electrophoresis with an ethidium bromide stain and ultraviolet illumination.

Another biochemical feature is the expression of cell surface markers that result in the early phagocytic recognition of apoptotic cells by adjacent cells, permitting quick phagocytosis with minimal compromise to the surrounding tissue. This is achieved by the movement of the normal inward-facing phosphatidylserine of the cell's lipid bilayer to expression on the outer layers of the plasma membrane (Bratton *et al.,* 1997). Although externalization of phosphatidylserine is a well-known recognition ligand for phagocytes on the surface of the apoptotic cell, recent studies have shown that other proteins are also be exposed on the cell surface during apoptotic cell clearance. These include Annexin V and calreticulin.

Annexin V is a recombinant phosphatidylserine-binding protein that interacts strongly and specifically with phosphatidylserine residues and can be used for the detection of apoptosis (Van Engeland *et al.,* 1998; Arur *et al.,* 2003). Calreticulin is a protein that

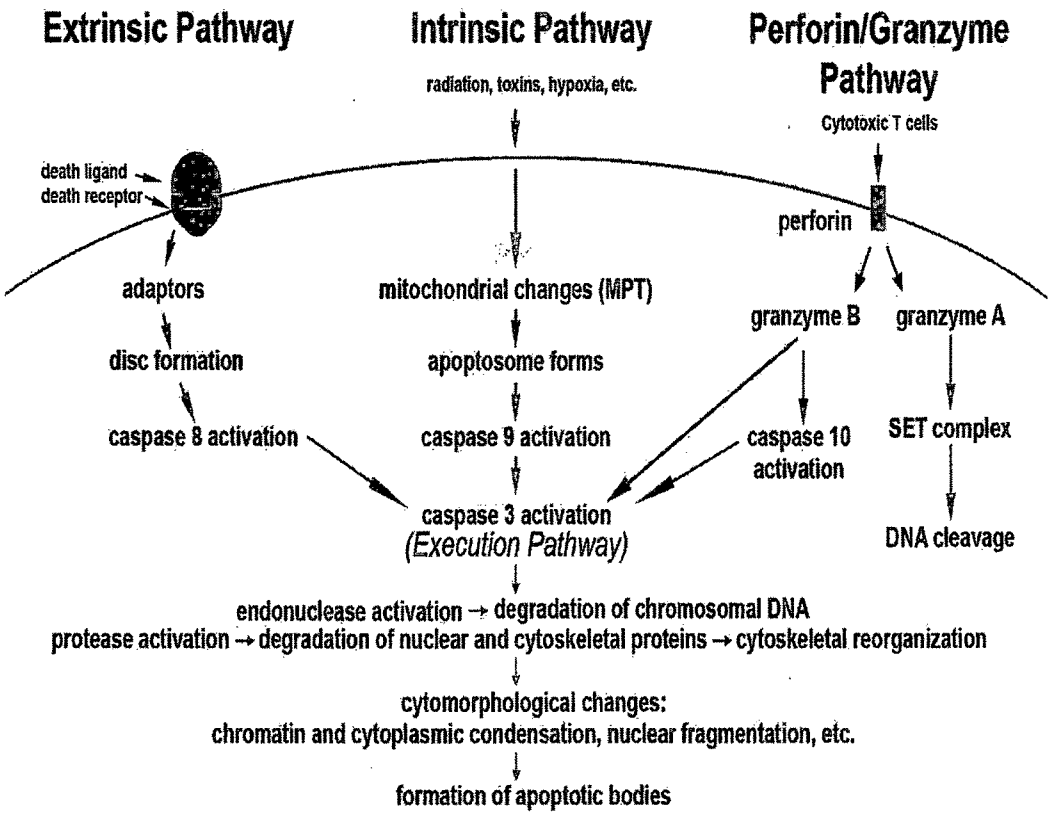
binds to an LDL-receptor related protein on the engulfing cell and is suggested to cooperate with phosphatidylserine as a recognition signal (Gardai *et al.*, 2005). The adhesive glycoprotein, thrombospondin-1, can be expressed on the outer surface of activated microvascular endothelial cells and, in conjunction with $CD^{2+}36$, caspase-3-like proteases and other proteins, induce receptor-mediated apoptosis (Jimenez *et al.*, 2000).

1.2.4 Molecular mechanism of apoptosis

The mechanisms of apoptosis are highly complex and sophisticated, involving an energy-dependent cascade of molecular events (Figure 1.4). To date, research indicates that there are two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. However, there is now evidence that the two pathways are linked and that molecules in one pathway can influence the other (Igney and Krammer, 2002). There is an additional pathway that involves T-cell mediated cytotoxicity and perforin-granzyme-dependent killing of the cell. The perforin/granzyme pathway can induce apoptosis via either granzyme B or granzyme A. The extrinsic, intrinsic, and granzyme B pathways converge on the same terminal, or execution pathway. This pathway is initiated by the cleavage of caspase-3 and results in DNA fragmentation, degradation of cytoskeletal and nuclear proteins, cross-linking of proteins, formation of apoptotic bodies, expression of ligands for phagocytic cell receptors and finally uptake by phagocytic cells. The granzyme A pathway activates a parallel, caspase-independent cell death pathway via single stranded DNA damage (Martinvalet *et al.*, 2005).

Caspases have proteolytic activity and are able to cleave proteins at aspartic acid residues, although different caspases have different specificities involving recognition of neighboring amino acids. Once caspases are initially activated, there seems to be an irreversible commitment towards cell death. To date, 14 major caspases have been identified and broadly categorized into initiators (caspase-2,-8,-9,-10), effectors or executioners (caspase-3,-6,-7) and inflammatory caspases (caspase-1,-4,-5) (Cohen, 1997). The other caspases that have been identified include caspase-11, which is

reported to regulate apoptosis and cytokine maturation during septic shock, caspase-12, which mediates endoplasmic-specific apoptosis and cytotoxicity by amyloid-β, caspase-13, which is suggested to be a bovine gene, and caspase-14, which is highly expressed in embryonic tissues but not in adult tissues (Hu *et al.*, 1998; Nakagawa *et al.*, 2000, Koenig *et al.*, 2001; Kang *et al.*, 2002).



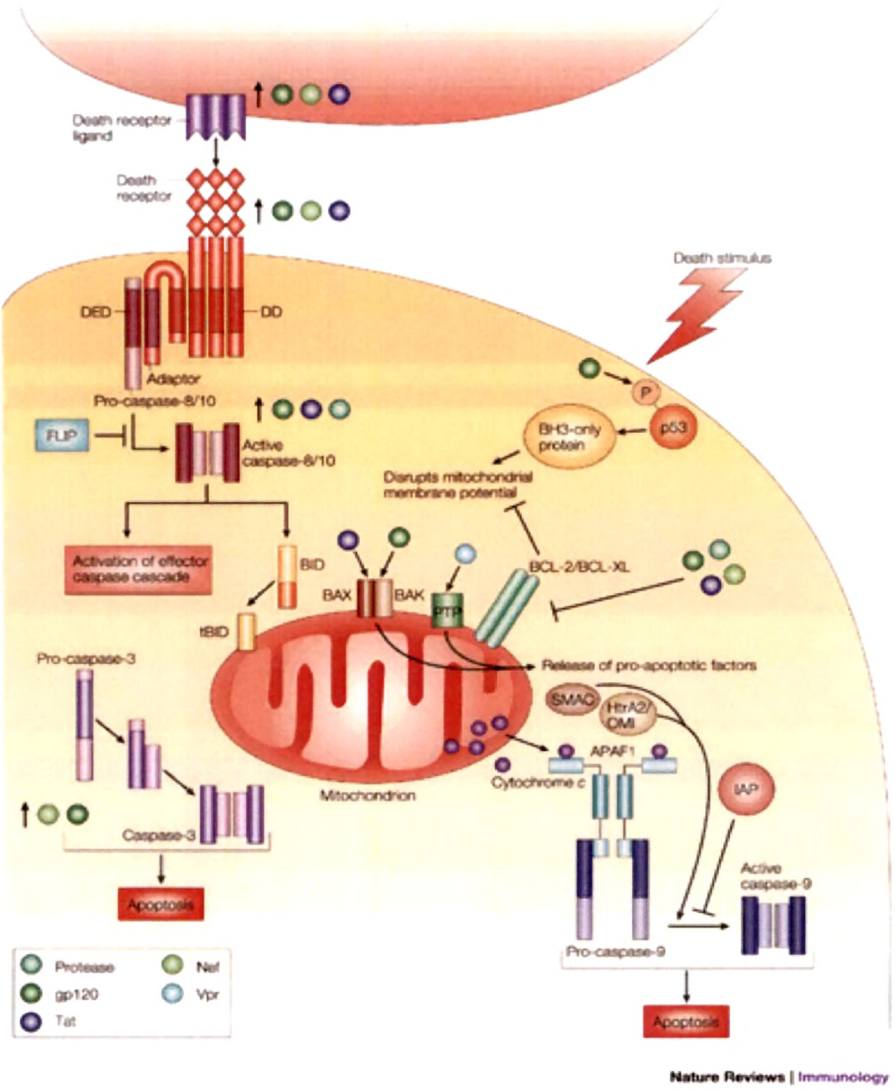
(Elmore, 2007)

Figure 1.4 Schematic representation of apoptotic events. The two main pathways of apoptosis are extrinsic and intrinsic as well as a perforin/granzyme pathway. Each requires specific triggering signals to begin an energy-dependent cascade of molecular events. Each pathway activates its own initiator caspase (8, 9, 10) which in turn will activate the executioner caspase-3. However, granzyme A works in a caspase-independent fashion. The execution pathway results in characteristic cytomorphological features including cell shrinkage, chromatin condensation, formation of cytoplasmic blebs and apoptotic bodies and finally phagocytosis of the apoptotic bodies by adjacent parenchymal cells, neoplastic cells or macrophages.

1.2.4.1 Extrinsic or death receptor-mediated pathway

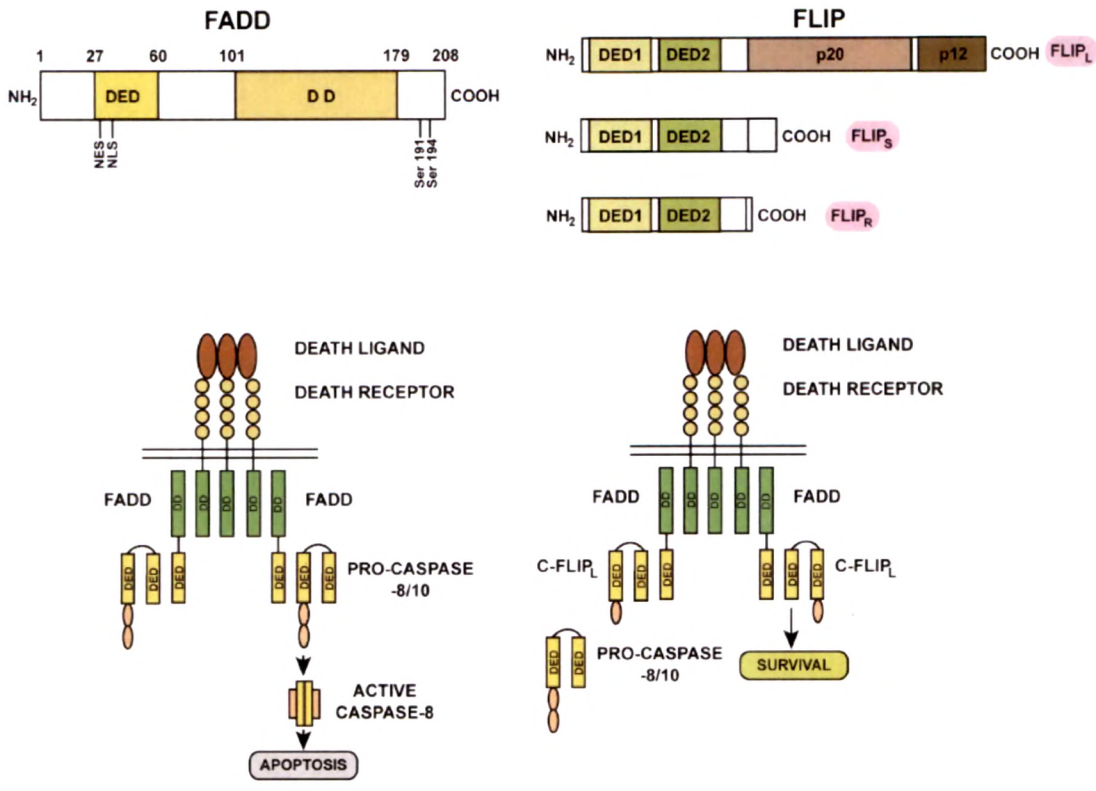
Extrinsic signaling at the cell surface can be initiated by aggregation of Fas receptors when they bind to the multivalent Fas Ligand (FasL). Both the death receptor (DR) and the ligand exist as trimers and the 'super clustering' of DRs following ligation is required to promote aggregation of procaspase 8 molecules within the DISC (Fanzo *et al.,* 2003). This aggregation brings the cytoplasmic domains of the membrane receptors into close proximity and induces a conformational change that allows the assembly of a signaling complex, the death inducing signaling complex (DISC), at the cytoplasmic tail of the receptors. The DISC comprises the receptors and ligand as well as an "adaptor" protein, Fas associated death domain protein (FADD), that binds through its C-terminal DD to the ligand-bound receptor and recruits procaspase-8. Procaspase-8 in turn binds to the DED of FADD via its own N-terminal DED domains. As a consequence of DISC formation at ligand-bound receptors, several molecules of procaspase-8 are brought into close proximity, resulting in high local concentration of procaspase-8 (Figures 1.5 and 1.6).

One hypothesis suggests that the low intrinsic activity of procaspase-8 allows the procaspase-8 zymogens to cleave and activate each other (induced proximity activation; Hengartner, 2000). Induced proximity activation has also been proposed for human caspase-2 and nematode CED-3 (Hengartner, 2000). However, other studies have suggested that the activation of caspase-8 requires dimerization (Boatright *et al.,* 2003). Active caspase-8 heterotetramers are released from death inducing signalling complex (DISC) and are free to cleave and activate the effector caspase, caspase-3. In some cells caspase-8 leads to an amplification loop that involves caspase-8 cleavage of the Bcl-2 protein family member, Bid. When Bid is cleaved it can induce Bax-mediated release of cytochrome c from the mitochondria, further committing the cell to the apoptosis fate.



(Gougeon and Kroemer, 2003)

Figure 1.5 The extrinsic pathway is initiated by the binding of tumour-necrosis factor (TNF)-family death-receptor ligands to their cognate receptors. Through their death domains (DDs), multimerized receptors interact with the DDs of adaptor proteins, which also contain death-effector domains (DEDs) that facilitate binding to pro-caspase-8 and/or pro-caspase-10 to form the death-inducing signal complex (DISC). As part of the DISC, the pro-caspases are cleaved into their active forms and initiate the intrinsic pathway of apoptosis. BID (BH3-interacting domain death agonist) is then cleaved to produce truncated (t)BID and the effector caspase cascade is activated. Death-receptor-induced apoptosis can be blocked by FLIP (FLICE-like inhibitory protein), which inhibits the proteolytic processing of caspase-8.



(Pasquini *et al.*, 2006)

Figure 1.6 Regulation of Apoptosis by FADD and FLIP proteins. *Left, top*: the structure of FADD protein, with two boxes indicating one DED domain and one DD domain, is shown. The numbers indicate the amino acid residue. Within the DED domain, nuclear export sequence (NES) and nuclear localization sequence (NLS) have been identified: they determine the nuclear localization of FADD either in the nucleus or in the cytoplasm. In the COOH terminal site two serine residues (Ser 191 and Ser 194), essential for FADD function, are indicated. *Right, top*: the structure of the three c-FLIP isoforms, $FLIP_L$, $FLIP_S$ and $FLIP_R$ with their structural domains is shown.

1.2.4.2 Perforin/granzyme pathway

T-cell mediated cytotoxicity is a variant of type IV hypersensitivity where sensitized $CD^{2+}8^{+}$ cells kill antigen-bearing cells. These cytotoxic T lymphocytes (CTLs) are able to kill target cells via the extrinsic pathway and the FasL/FasR interaction is the predominant method of CTL-induced apoptosis (Brunner *et al.*, 2003). However, they are also able to exert their cytotoxic effects on tumor cells and virus-infected cells via a novel pathway that involves secretion of the transmembrane pore-forming molecule

perforin with a subsequent exophytic release of cytoplasmic granules through the pore and into the target cell (Trapani and Smyth, 2002). The serine proteases granzyme A and granzyme B are the most important component within the granules (Pardo *et al.*, 2004).

Granzyme B will cleave proteins at aspartate residues and will therefore activate procaspase-10 and can cleave factors like ICAD (Inhibitor of Caspase Activated DNAse) (Sakahira *et al.*, 1998). Reports have also shown that granzyme B can utilize the mitochondrial pathway for amplification of the death signal by specific cleavage of Bid and induction of cytochrome *c* release (Barry and Bleackley, 2002; Russell and Ley, 2002). However, granzyme B can also directly activate caspase-3. In this way, the upstream signaling pathways are bypassed and there is direct induction of the execution phase of apoptosis.

It is suggested that both the mitochondrial pathway and direct activation of caspase-3 are critical for granzyme B-induced killing (Goping *et al.*, 2003). Recent findings indicate that this method of granzyme B cytotoxicity is critical as a control mechanism for T cell expansion of type 2 helper T (Th2) cells (Devadas *et al.*, 2006). Moreover, findings indicate that neither death receptors nor caspases are involved with the T cell receptor-induced apoptosis of activated Th2 cells because blocking their ligands has no effect on apoptosis. On the other hand, Fas-Fas ligand interaction, adapter proteins with death domains and caspases are all involved in the apoptosis and regulation of cytotoxic Type 1 helper cells whereas granzyme B has no effect.

Granzyme A is also important in cytotoxic T cell induced apoptosis and activates caspase independent pathways. Once in the cell, granzyme A activates DNA nicking via DNAse NM23-H1, a tumor suppressor gene product (Fan *et al.*, 2003). This DNAse has an important role in immune surveillance to prevent cancer through the induction of tumor cell apoptosis. The nucleosome assembly protein SET normally inhibits the NM23-H1 gene. Granzyme A protease cleaves the SET complex thus releasing inhibition of NM23-H1, resulting in apoptotic DNA degradation. In addition

to inhibiting NM23-H1, the SET complex has important functions in chromatin structure and DNA repair. The proteins that make up this complex (SET, ApeI, pp32, and HMG2) seem to work together to protect chromatin and DNA structure (Lieberman and Fan, 2003). Therefore, inactivation of this complex by granzyme A most likely also contributes to apoptosis by blocking the maintenance of DNA and chromatin structure integrity.

1.2.4.3 Intrinsic or Mitochondria mediated pathway

The intrinsic signaling pathways that initiate apoptosis involve a diverse array of non-receptor mediated stimuli that produce intracellular signals that act directly on targets within the cell and are mitochondrial initiated events (Figure 1.7). The stimuli that initiate the intrinsic pathway produce intracellular signals that may act in either a positive or negative fashion. Negative signals involve the absence of certain growth factors, hormones and cytokines that can lead to failure of suppression of death programs, thereby triggering apoptosis. In other words, there is the withdrawal of factors, loss of apoptotic suppression, and subsequent activation of apoptosis. Other stimuli that act in a positive fashion include, but are not limited to, radiation, toxins, hypoxia, hyperthermia, viral infections, and free radicals.

All of these stimuli cause changes in the inner mitochondrial membrane that results in an opening of the mitochondrial permeability transition (MPT) pore, loss of the mitochondrial transmembrane potential and release of two main groups of normally sequestered pro-apoptotic proteins from the intermembrane space into the cytosol (Saelens *et al.*, 2004). The first group consists of cytochrome c, Smac/DIABLO, and the serine protease HtrA2/Omi (Cai *et al.*, 1998; Du *et al.*, 2000; Loo *et al.*, 2002; Garrido *et al.*, 2005). These proteins activate the caspase-dependent mitochondrial pathway. Cytochrome c binds and activates Apaf-1 as well as procaspase-9, forming an "apoptosome" (Hill *et al.*, 2004).

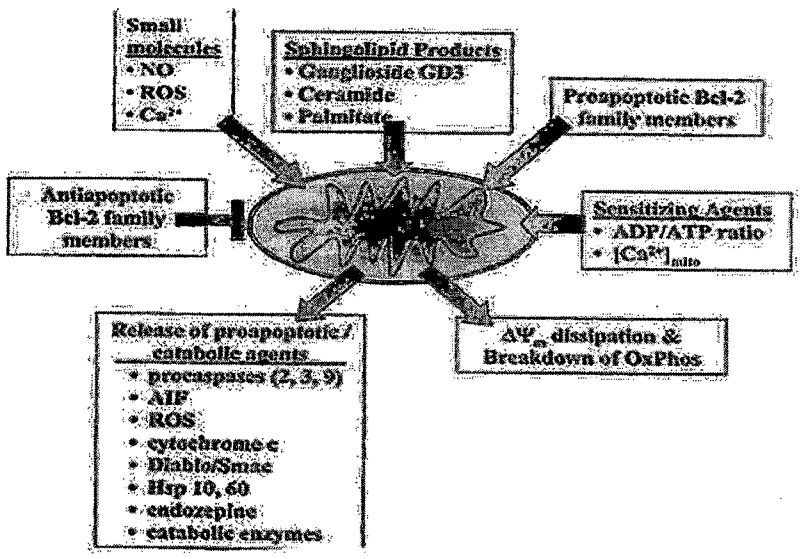
The clustering of procaspase-9 in this manner leads to caspase-9 activation. Smac/DIABLO and HtrA2/Omi are reported to promote apoptosis by inhibiting IAP

(inhibitors of apoptosis proteins) activity (van Loo *et al.,* 2002; Schimmer, 2004). Additional mitochondrial proteins have also been identified that interact with and suppress the action of IAP however gene knockout experiments suggest that binding to IAP alone may not be enough evidence to label a mitochondrial protein as "pro-apoptotic" (Ekert and Vaux, 2005).

The second group of pro-apoptotic proteins, AIF, endonuclease G and CAD, are released from the mitochondria during apoptosis, but this is a late event that occurs after the cell has committed to die. AIF leads to early form of nuclear condensation which is referred to as "stage I" condensation (Susin *et al.,* 2000). Endonuclease G also translocates to the nucleus where it cleaves nuclear chromatin to produce oligonucleosomal DNA fragments (Li *et al.,* 2001). AIF and endonuclease G both function in a caspase-independent manner. CAD is subsequently released from the mitochondria and translocates to the nucleus where, after cleavage by caspase-3, it leads to oligonucleosomal DNA fragmentation and a more pronounced and advanced chromatin condensation (Enari *et al.,* 1998). This later and more pronounced chromatin condensation is referred to as "stage II" condensation (Susin *et al.,* 2000).

The control and regulation of these apoptotic mitochondrial events occurs through members of the Bcl-2 family of proteins (Cory and Adams, 2002). The tumor suppressor protein p53 has a critical role in regulation of the Bcl-2 family of proteins, however the exact mechanisms have not yet been completely elucidated (Schuler and Green, 2001). The Bcl-2 family of proteins governs mitochondrial membrane permeability and can be either pro-apoptotic or anti-apoptotic. To date, a total of 25 genes have been identified in the Bcl-2 family. Some of the anti-apoptotic proteins include Bcl-2, Bcl-x, Bcl-XL, Bcl-XS, Bcl-w, BAG, and some of the pro-apoptotic proteins include Bcl-10, Bax, Bak, Bid, Bad, Bim, Bik, and Blk. These proteins have special significance since they can determine if the cell commits to apoptosis or aborts the process. Bcl-2 family members are critical regulators of mitochondrial-dependent apoptosis, whereby pro- and anti-apoptotic family members control the permeability of the mitochondrial outer membrane to apoptogenic proteins. The initial events are thought to be the down-regulation of the anti-apoptotic mitochondrial membrane

protein Bcl-2, and the activation of the pro-apoptotic Bcl-2 family member, Bax. Bax is activated directly by the p53 tumour suppressor protein following stress induction, or indirectly through the p53-activation of the Bcl-2 pro-apoptotic members, phorbol-12-myristate-13-acetate-induced protein 1 (Noxa) and PUMA, or thorough p53-independent mechanisms. The activation of Bax results in its movement from the cytosol to the mitochondrial membrane, where it oligomerises and embeds itself in the membrane to create a pore. The down-regulation of Bcl-2 is required to prevent it blocking Bax oligomerization. The formation of the Bax pore, as well as the consequent loss of mitochondrial membrane potential, causes proteins like cytochrome c and AIF to leak out of the mitochondria into the cytosol. Once in the cytosol, cytochrome c forms a complex with Apaf-1 (apoptosis protease activating factor) and caspase-9 called the apoptosome. The Apaf-1 activation of caspase-9 within the apoptosome is a key event that triggers the activation of the caspase cascade, including caspases-3 and –7, which execute the cell death programme.



(Olson and Kornbluth, 2011)

Figure 1.7 Mitochondrial involvement in apoptosis. A number of agents have been implicated in modulating mitochondrial integrity. Bcl- ~~(Olson and Kornbluth, 2001)~~ and antiapoptotic), calcium, reactive oxygen species, and sphingolipid precursors and metabolites can all affect mitochondrial membrane depolarization. Intrinsic factors such as mitochondrial $[Ca^{2+}]$ and ADP/ATP ratio may affect mitochondrial sensitivity to externally applied stimuli. Consequences of mitochondrial involvement in apoptosis include release of catabolic and apoptogenic factors, and breakdown of oxidative phosphorylation (OxPhos).

1.2.4.3.1 Apoptosome formation and pro-caspase 9 activation

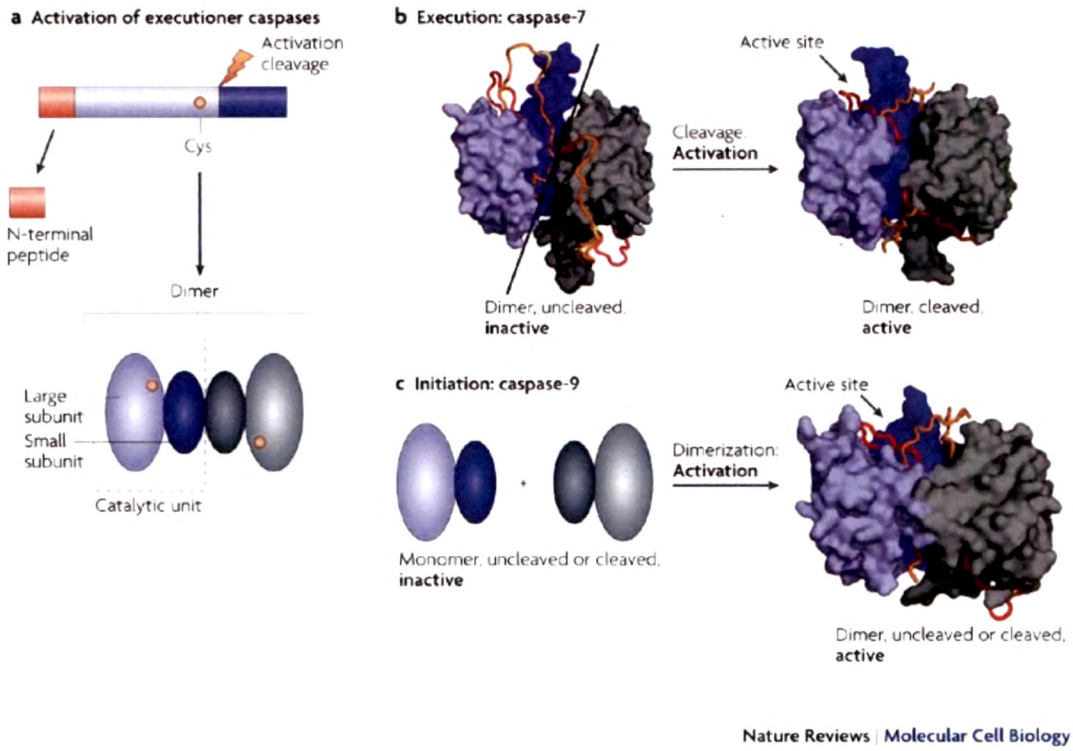
Apaf-1 is a multidomain protein that consists of three functional regions, an N-terminal caspase-recruitment domain (CARD), a nucleotide-binding and oligomerization domain (NOD; also referred to as NB-ARC) and a string of WD40 repeats in the C-terminal half of the protein. CARD domain is involved in homotypic interaction with CARD domain of caspase-9. The WD40 repeats are responsible for the binding of cytochrome c. The NB-ARC domain forms the centre of oligomerization and, therefore, apoptosome formation. In the absence of an apoptotic signal, Apaf-1 exists in a monomeric form. It is thought that in this form the WD40-repeat domains restrain Apaf-1 in an autoinhibited state. A likely scenario is that during the expression of Apaf-1, (d)ATP is incorporated into the latent (locked) form to generate the centre of the autoinhibited model (Kim *et al.,* 2005; Yu *et al.,* 2005). The binding of cytochrome c increases Apaf-1 affinity for dATP/ATP by about 10-fold, perhaps by opening up the nucleotide binding site or stabilizing the bound nucleotide to Apaf-1 (Jiang and Wang, 2000). The binding of nucleotide to the Apaf-1/cytochrome c complex triggers its oligomerization to form the apoptosome, a multimeric Apaf-1 and cytochrome c complex (Zou *et al.,* 1999). The CARD domains of Apaf-1 become exposed in the apoptosome, which subsequently recruit multiple procaspase-9 molecules to the complex and facilitate their autoactivation. Unlike the executioner caspases, the zymogen of caspase-9 is primarily a monomer at physiological concentration, a property it shares with other initiator caspases. Only the caspase-9 bound to the apoptosome is able to efficiently cleave and activate downstream executioner caspases such as caspase-3 (Rodriguez and Lazebnik, 1999).

1.2.3.4 Execution of apoptosis by caspases

The extrinsic and intrinsic pathways both end at the point of the execution phase, considered the final pathway of apoptosis. It is the activation of the execution caspases that begins this phase of apoptosis. Execution caspases activate cytoplasmic endonuclease, which degrades nuclear material, and proteases that degrade the nuclear and cytoskeletal proteins. Caspase-3, caspase-6, and caspase-7 function as effector or "executioner" caspases (Slee *et al.,* 2001).

Caspase-3 is considered to be the most important of the executioner caspases and is activated by any of the initiator caspases (caspase-8, caspase-9, or caspase-10). Caspase-3 specifically activates the endonuclease CAD. In proliferating cells CAD is complexed with its inhibitor, ICAD. In apoptotic cells, activated caspase-3 cleaves ICAD to release CAD (Sakahira *et al.,* 1998). CAD then degrades chromosomal DNA within the nuclei and causes chromatin condensation. Caspase-3 also induces cytoskeletal reorganization and disintegration of the cell into apoptotic bodies. Gelsolin, an actin binding protein, has been identified as one of the key substrates of activated caspase-3. Gelsolin will typically act as a nucleus for actin polymerization and will also bind phosphatidylinositol biphosphate, linking actin organization and signal transduction. Caspase-3 will cleave gelsolin and the cleaved fragments of gelsolin, in turn, cleave actin filaments in a calcium independent manner. These results in disruption of the cytoskeleton, intracellular transport, cell division, and signal transduction (Kothakota *et al.,* 1997).

Phagocytic uptake of apoptotic cells is the last component of apoptosis. Phospholipid asymmetry and externalization of phosphatidylserine on the surface of apoptotic cells and their fragments is the hallmark of this phase. Although the mechanism of phosphatidylserine translocation to the outer leaflet of the cell during apoptosis is not well understood, it has been associated with loss of aminophospholipid translocase activity and nonspecific flip-flop of phospholipids of various classes (Bratton *et al.,* 1997). Research indicates that Fas, caspase-8, and caspase-3 are involved in the regulation of phosphatidylserine externalization on oxidatively stressed erythrocytes however caspase-independent phosphatidylserine exposure occurs during apoptosis of primary T lymphocytes (Ferraro-Peyret *et al.,* 2002; Mandal *et al.,* 2005). The appearance of phosphotidylserine on the outer leaflet of apoptotic cells then facilitates noninflammatory phagocytic recognition, allowing for their early uptake and disposal (Fadok *et al.,* 2001). This process of early and efficient uptake with no release of cellular constituents, results in essentially no inflammatory response.



(Riedl and Salvesen, 2007)

Figure 1.8 Activation of executioner caspases a | Activation of executioner caspases. Caspases are initially expressed as single-chain proteins that undergo an activation cleavage. An executioner caspase is typically cleaved twice, leading to the release of a short N-terminal peptide. The actual activation cleavage divides the catalytic unit into a large and small subunit. The position of the active-site Cys residue is indicated in orange.

b | Surface rendering of an executioner caspase, caspase-7, before and after activation cleavage. The same colour code as in panel a is used, and important loop regions are shown as ribbons. Cleavage releases strains on surface loops (red and orange) and the chains rearrange. The newly formed termini of the large and small subunits (orange) interact with each other across the other catalytic unit and with the red loops to align the substrate-binding pockets at the bottom of the active-site cleft. This results in a highly active caspase (right).

c | Although they can be cleaved (as revealed, for example, in their crystal structures), initiator caspases, such as caspase-9, show full activity in their uncleaved forms, which could be due to the long linker loops in between subunits. Their activity is regulated by dimerization instead of by cleavage. Initiator caspases exist as inactive monomers (left). Dimerization allows for the formation of a productive active site, shown here in the structure of cleaved, dimeric caspase-9 (right). Interestingly, only one of the two sites adopts the active form in the crystal structure of caspase-9.

1.2.4 The discovery of metacaspases and paracaspases

At the end of 2000, distant caspase relatives were discovered *in silico* in plants, fungi, and protozoa and were designated metacaspases (Uren *et al.,* 2000). The sequences of previously found caspase like proteins (paracaspases) in metazoans and in the slime mold *D. discoideum* had been used in an iterative PSI-BLAST search of plant-expressed sequence tags (Aravind *et al.,* 1999). Paracaspases contain a prodomain consisting of a death domain and one or two Ig domains, whereas two types of metacaspases can be distinguished (Figure 1.9 A). Type I metacaspases have an N-terminal extension reminiscent of the prodomain in initiator and inflammatory caspases. Type II metacaspases lack such a prodomain but harbor a linker region between the putative large and small subunits (Uren *et al.,* 2000; Vercammen *et al.,* 2004). Both meta- and paracaspases contain a conserved catalytic His/Cys dyad, and structure predictions show that they bear the core of the caspase/hemoglobinase fold (Figure 1.9 B), which is the determining structural feature of all clan CD^{2+} Cys proteases (Rawlings and Barrett, 1993; Aravind and Koonin, 2002).

1.2.4.1 Metacaspases and cell death

In the Arabidopsis genome, nine metacaspase genes are present: three of type I (Arabidopsis thaliana metacaspase 1 [AtMC1] to AtMC3) and six of type II (AtMC4 to AtMC9; Vercammen *et al.,* 2004). Upon overproduction in Escherichia coli, type II metacaspases autoprocess and display a Cys-dependent proteolytic activity against synthetic P1-Arg substrates, whereas AtMC9 also cleaves P1-Lys substrates, albeit with low efficiency (Vercammen *et al.,* 2004, 2006; Watanabe and Lam, 2005). Type I metacaspases from Arabidopsis do not autoprocess upon recombinant overproduction and, like mammalian initiator caspases, possibly require induced oligomerization within an activation platform (Fuentes-Prior and Salvesen, 2004).

A yeast strain (Saccharomyces cerevisiae) with a disrupted YCA1 gene (Δyca1) was also shown to be threefold less sensitive to H_2O_2, and □5% of the cells escaped from aging-related cell death (Madeo *et al.,* 2002). Interestingly, Extracts of H_2O_2-treated YCA1-overproducing yeast were highly active toward the synthetic caspase substrates

1.2.4.2 Paracaspases and cell death

Paracaspases (human: MALT1) are related to caspases present in animals and slime mold, in contrast to metacaspases, which are present in plants, fungi, and "protists"(Uren *et al.,* 2000). Paracaspases are more similar to caspases than metacaspases are, indicating that this group of proteases diverged from caspases from a common metacaspase ancestor.

Paracaspase has been first identified in a recurrent t(11;18)(q21;q21) chromosomal translocation associated with a subset of MALT lymphoma. This leads to a fusion oncoprotein consisting of the carboxyl terminus of MALT1 and the amino terminus of c-IAP2. Genetic ablation of the paracaspase gene is mice and biochemical studies have shown that paracaspase is a crucial protein for T and B lymphocytes activation. It has an important role in the activation of the transcription factor NF-κB, in the production of interleukin-2 (IL-2) and in T and B lymphocytes proliferation (Ruefli-Brasse *et al.,* 2003; Ruland *et al.,* 2003). In addition, a role for paracaspase has been shown in the innate immune response mediated by the zymosan receptor Dectin-1 in macrophages and dendritic cells, and in response to the stimulation of certain G protein-coupled receptors (Wegener and Krappmann, 2007). Sequence analysis proposes that paracaspase has a N-terminal death domain, two central immunoglobulin-like domains involved in the binding to the B-cell lymphoma 10 (Bcl-10) protein and a caspase-like domain. However, alleged paracaspase of *D. discoideum* is a surprising element in this phylogenetic distribution because slime molds belong to the Protozoa kingdom. Phylogenetic analysis of the sequence of its putative catalytic p20 subunit reveals that it is almost equally related to that of caspases, metacaspases, paracaspases, and their bacterial homologues, making its classification as a separate paracaspase not well founded. Also, its prodomain lacks a death domain and Ig domains, which is typical of animal paracaspases. Therefore, it is tempting to classify the *D. discoideum* protease as a metacaspase rather than a paracaspase (Cavalier-Smith, 2004; Vercammen *et al.,* 2007).

1.3 Paraptotic cell death or caspase-independent PCD

PCD can occur in complete absence of caspases, and other noncaspase proteases have been described to be able to execute PCD. It has become clear that inhibition of caspase activation does not necessarily protect against cell death stimuli but rather can even enhance underlying caspase independent death programs (Sperandio *et al.,* 2000). Most commonly observed caspase independent cell death is paraptosis. The features of paraptosis differ from those of apoptosis and involve cytoplasmic vacuolation, mitochondrial swelling and absence of caspase activation or typical nuclear changes, including pyknosis and DNA fragmentation (Sperandio *et al.,* 2000; Wyllie and Golstein, 2001).

Table 1.1 Morphological features of Apoptosis, Necrosis and paraptosis: A comparison between three major forms of cell death.

Morphological features			
Parameters	**Apoptosis**	**Necrosis**	**Paraptosis**
Outset	Shrinking of cytoplasm, condensation of nucleus.	Swelling of cytoplasm and mitochondria.	Cytoplasmic vacuolisation
Plasma membrane	Blebbing of plasma membrane without loss of integrity	Loss of membrane integrity	–
Nuclear chromatin	Aggregation of chromatin at the nuclear membrane.	Karyolysis	Chromatin condensation
Cytoplasmic organelles	Last stage swelling	Very early swelling	Last stage swelling in case of ER and mitochondria
Mitochondria	Mitochondria become leaky due to pore formation involving proteins of the bcl-2 family.	Disintegration (swelling) of organelles	Mitochondria become leaky due to pore formation involving proteins of the bcl-2 family
Fate of cell	Formation of apoptotic body	Swelling and later disintegration	Absence of apoptotic body formation

Table 1.2 Biochemical features of Apoptosis

Biochemical features			
Parameters	**Apoptosis**	**Necrosis**	**Paraptosis**
Regulation	Tightly regulated process involving activation and enzymatic steps.	Loss of regulation of ion homeostasis.	_
Energy input	Energy (ATP)-dependent (active process, does not occur at 4°C)	No energy requirement (passive process, also occurs at 4°C)	_
DNA	Non-random mono- and oligonucleosomal length fragmentation of DNA (Ladder pattern after agarose gel electrophoresis)	Random digestion of DNA (smear of DNA after agarose gel electrophoresis)	Large scale (>50kb fragments)?
Timing	Pre lytic DNA fragmentation	Post lytic DNA fragmentation (= late event in cell death)	_
Biochemical events	Activation of caspase cascade (mainly caspase-3)	Absence of caspase-3 activation but caspase 1 activation may occur	AIF mediated but caspase 1 activation may occur
PARP cleavage	Presence of PARP cleavage	Presence of PARP cleavage	_

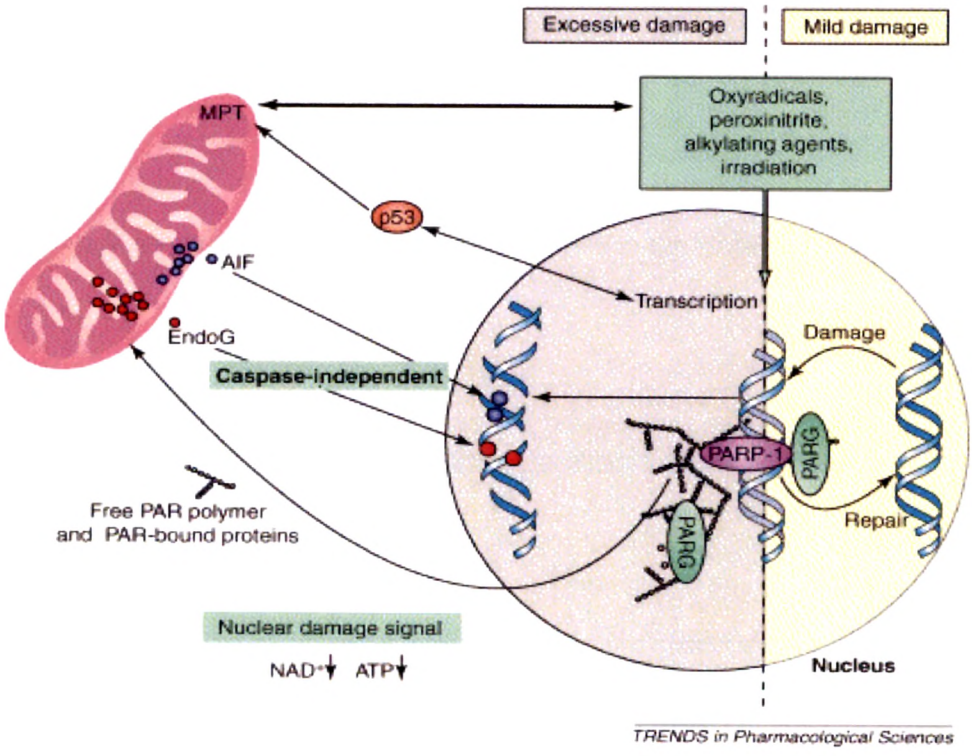
Table 1.3 Physiological impact of Apoptosis, Necrosis and paraptosis

Physiological impact			
Parameters	**Apoptosis**	**Necrosis**	**Paraptosis**
Extent	Localized effect that destroys individual cells	Affects groups of contiguous cells	_
Induction	Physiological: Induced by physiological stimuli (lack of growthfactors, changes in hormonal environment).	Physicochemical: Evoked by non-physiological disturbances (complement attack, lytic viruses, hypothermia, metabolic poisons, hypoxia, ischemica)	_
Phagocytosis	Phagocytosis by adjacent cells or macrophages	Phagocytosis by macrophages	_
Immune system	No inflammatory response	Significant inflammatory response.	_

(Table contents from Susan *et al.*, 2005, Zong *et al.*, 2006, Wang *et al.*, 2004, Sperandio *et al.*, 2000).

There is increasing evidence that this alternative, non-apoptotic PCD exists in parallel with apoptosis. For instance, T9 glioma cells expressing membrane macrophage colony-stimulating factor were killed by polymorphonuclear leukocytes and macrophages with vacuolization that begins with progressive swelling of mitochondria and the endoplasmic reticulum (ER).

Paraptosis has been described to be mediated by mitogen activated protein kinases (Sperandio *et al.*, 2004) and can be triggered by the TNF receptor family member TAJ/TROY (Wang *et al.*, 2004), the insulin like growth factor I receptor (Sperandio *et al.*, 2004) and poly(ADP-ribose) polymerase (PARP) via DNA damage (Fig. 1.10). PARP mediated paraptosis involves utilization of NAD^+ and depletion in energy levels, change in mitochondrial membrane potential, release of AIF and finally leading to cell death.



(Hong *et al.*, 2004)

Figure 1.10 Poly(ADP-ribose) polymerase 1 (PARP-1)

Paraptosis mediated by mitogen activated protein kinases (MAPKs) is shown to be inhibited by AIP-1/Alix. The inhibition of cell death by AIP-1/Alix is specific for paraptosis, it does not affect apoptosis (Sperandio *et al.,* 2004). Engagement of at least two signaling pathways triggered by IGFIR, the MAPK/ERK and JNK pathways, occurred in paraptosis. Involvement of MAPK in paraptosis is surprising since this pathway has typically been associated with cell survival or proliferation rather than cell death (Chang and Karin, 2001). However, other groups have demonstrated that the activation of ERKs is necessary for cell death in different paradigms, such as neuronal cell death induced by glutamate (Mukherjee and Pasinetti, 2001). Further work is required to characterize the upstream activators and the downstream targets of MAPK involved in nonapoptotic PCD, and to discern the MAPK dependent signals that distinguish a trophic response from a PCD response.

Recent study has demonstrated that taxol (a potent anti cancer drug) induced paraptosis required neither protein synthesis nor the participation of MEK, JNK, and p38, which was different from the insulin like growth factor I receptor (IGFIR) induced paraptosis. Taxol induced morphological changes and motility of endoplasmic reticulum; massive vacuolization is observed which could be due to pore formation in ER membrane by Bcl-XL or BK channel activation (Sun *et al.,* 2010). Accumulating evidence now suggest that necrosis like apoptosis can be executed by regulated mechanisms. Such cell death is termed as necroptosis. Necroptosis involves extensive network of genes and different pathways activated by RIP kinase (Hitomi *et al.,* 2008).

Paraptosis is mainly mediated by three proteins among others: Poly(ADP-ribose) polymerase (PARP), Poly(ADP-ribose) glycohydrolase (PARG) and Apoptosis inducing factor (AIF).

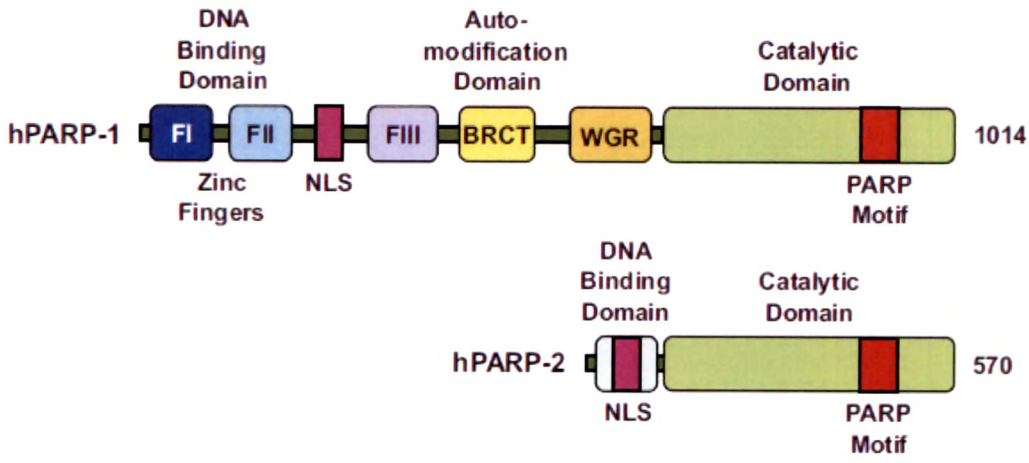
1.3.1 Poly(ADP-ribose) polymerase (PARP)

Nuclear processes involving access to or modification of the genome, such as transcription and DNA repair, require a host of structural and regulatory proteins.

Poly(ADP-ribose) polymerase-1 (PARP-1), a ubiquitous and abundant nuclear protein and a member of the PARP family, has a number of distinct biochemical activities that make it well suited for both structural and regulatory roles across the genome (Hassa and Hottiger, 2008; Kim *et al.,* 2005; Schreiber *et al.,* 2006). PARP-1 can bind to various DNA structures and nucleosomes, and it possesses an NAD^+-dependent catalytic activity that synthesizes a negatively charged polymer on target proteins called poly(ADP-ribose) or PAR. Although historically studied in the context of DNA damage detection and repair, PARP-1 has more recently been linked to the regulation of chromatin structure and transcription, DNA methylation and imprinting, insulator activity, and chromosome organization alongwith cell death.

1.3.1.1 PARP-1 Structure and Biochemical Activities

PARP-1 is a highly conserved protein of 116 kDa (D'Amours *et al.,* 1999). Like many other chromatin- and transcription related proteins, it has a modular structure comprising multiple independently folded domains. The major functional units of PARP-1 are an amino-terminal DNA-binding domain (DBD), a central automodification domain (AMD), and a carboxyterminal catalytic domain (CD^{2+}) (Hakme *et al.,* 2008; Schreiber *et al.,* 2006) (Figure 1.11A). The DBD contains two Cys-Cys-His-Cys zinc fingers (FI/Zn1 and FII/Zn2) that mediate binding to DNA, a newly discovered third zinc binding domain (FIII/Zn3) that mediates interdomain contacts important for DNA-dependent enzyme activation (Langelier *et al.,* 2008, 2010), a nuclear localization signal (NLS), and a caspase-3 cleavage site (Hakme *et al.,* 2008; Schreiber *et al.,* 2006). The AMD contains a BRCT (BRCA1 C terminus) fold, which mediates protein-protein interactions (e.g., with DNA repair enzymes). The CD^{2+}, which is the most conserved domain across the PARP family, contains a PARP signature motif, which binds NAD^+, as well as a "WGR" motif, which is named after the most conserved amino acid sequence in the motif (Trp, Gly, Arg) and has an unknown function. The structures of these domains and motifs are shown in Figure 1.11B. Together, the structural and functional domains of PARP-1 confer the activities required for the broad range of functions of PARP-1 in the nucleus.



(Kraus, 2010)

Figure 1.11 Structural and Functional Organization of PARP-1 and PARP-2 (A) Schematic representation of human PARP-1 and PARP-2 with the functional domains noted in the text. (B) Structures of the six structural and functional domains in human PARP-1: FI (PDB 2DMJ), FII (PDB 2CS2), FIII (PDB 2RIQ), BRCT (PDB 2COK), WGR (PDB 2CR9), and catalytic domain.

1.3.1.2 Biochemistry of PARP-1 DNA Binding, Chromatin Binding, and Genomic Localization

Studies over the past few decades have shown that PARP-1 associates with chromatin in specific patterns that relate to its function (Kraus, 2008; Kraus and Lis, 2003; Tulin *et al.*, 2003). This association is driven by interactions with DNA, nucleosomes, or other chromatin associated proteins, which are not mutually reflected in a recent structure-based classification of PARP family members into three groups based on their catalytic domains: (1) PARPs 1-5, which are bona fide PARPs containing a conserved glutamate (Glu 988 in PARP-1) that defines the PARP catalytic activity; (2) PARPs 6–8, 10–12, and 14–16, which are confirmed or putative mARTs; and (3) PARPs 9 and 13, which lack key NAD^+-binding residues and the catalytic glutamate, and are likely to be inactive (Kleine *et al.*, 2008).

PARP family members localize to various cellular compartments, including the nucleus, cytoplasm, mitochondria, and vault particles, although the subcellular

localization and function of many of the PARPs are unknown (Ame *et al.,* 2004; Hassa and Hottiger, 2008; Cookson *et al.,* 1999). The primary nuclear PARPs are PARP-1, PARP-2 (the closest paralog to PARP-1), PARP-3, and tankyrases 1 and 2 (PARP-5a and -5b) (Ame *et al.,* 2004; Hakme *et al.,* 2008; Hassa and Hottiger, 2008; Schreiber *et al.,* 2006). PARP-1 binds to a variety of DNA structures, including single- and double-strand breaks, crossovers, cruciforms, and supercoils, as well as some specific double-stranded DNA sequences (Kraus, 2008; Kraus and Lis, 2003). PARP-1 also binds to nucleosomes in a specific manner, interacting with both DNA and histones at or near the dyad axis where the DNA enters and exits the nucleosome (Kim *et al.,* 2004). Finally, PARP-1 can interact with a wide variety of chromatin-associated proteins, including components of the transcription machinery, sequence-specific DNA-binding transcription factors, chromatin-modifying enzymes, and histone variants (e.g., macroH2A) (Kim *et al.,* 2005; Kraus, 2008; Kraus and Lis, 2003; Tulin *et al.,* 2003). Interactions with these proteins allow for indirect association of PARP-1 with chromatin. By binding to chromatin, PARP-1 can alter the structure of nucleosomes, as well as the composition or compaction state of chromatin (Kim *et al.,* 2004; Kraus, 2008; Kraus and Lis, 2003; Langelier *et al.,* 2010; Tulin *et al.,* 2003; Wacker *et al.,* 2007). This may occur through target protein modification by PARP-1's enzymatic activity, as well as competition for binding sites on nucleosomes. For example, PARP-1 may displace the linker histone H1 from nucleosomes by PARylating it or by competing for overlapping binding sites on the nucleosomes (Ju *et al.,* 2006; Kim *et al.,* 2004; Krishnakumar *et al.,* 2008).

A recent genomic localization study has shown that PARP-1 binds at the promoters of most actively transcribed genes (Krishnakumar *et al.,* 2008). The binding of PARP-1 at promoters correlates with the binding of Pol II, gene expression, and the presence of histone H3 lysine 4 trimethylation (H3K4me3), a histone modification that marks active promoters. PARP-1 also binds to chromatin outside of promoter regions, including enhancers (Krishnakumar *et al.,* 2008). In response to genotoxic stress, PARP-1 relocalizes to sites of DNA damage (i.e., nicks, breaks) (Haince *et al.,* 2008; Mortusewicz *et al.,* 2007). Whether this DNA damage-induced relocalization results in a global redistribution of PARP-1 away from promoters, as was shown recently for

the NAD^+-dependent chromatin regulator Sirt1 (Oberdoerffer *et al.,* 2008), remains to be determined. This is an attractive model that fits well with the global reduction in transcription observed in response to DNA damage.

1.3.1.3 Catalytic Activity, Binding Partners and Targets

PAR is a large, negatively charged polymer that functions as a posttranslational modification, as well as a free polymer. Most of the PAR in the cell is produced by the catalytic activity of PARP-1, which catalyzes the polymerization of ADP-ribose units from donor NAD^+ molecules on target proteins (D'Amours *et al.,* 1999). The ADP-ribose units are linked to each other via glycosidic ribose-ribose bonds, and the resulting PAR polymers may be linear or branched (D'Amours *et al.,* 1999).

PARP-1 catalytic activity is regulated through allosteric mechanisms involving a range of binding partners, including damaged DNA, histones, nucleosomes, and an assortment of nuclear proteins (D'Amours *et al.,* 1999; Kraus and Lis, 2003; Tulin *et al.,* 2003). PARP-1 catalytic activity is also regulated by posttranslational modifications; autoPARylation of PARP-1 inhibits its catalytic activity, while phosphorylation by Erk1/2 enhances its catalytic activity (Kauppinen *et al.,* 2006). PARP-1 catalytic activity may also be regulated by nicotinamide mononucleotide adenylyltransferase-1 (NMNAT-1), a nuclear NAD^+ synthase that interacts with PARP-1 and can produce NAD^+ locally for use by nuclear enzymes that require NAD^+, such as PARP-1 and Sirt1 (Kim *et al.,* 2004; Zhang *et al.,* 2009). PARP-1, which has many protein binding partners in the nucleus, has been identified as a component of a wide variety of protein complexes, including those that (1) repair DNA damage (e.g., condensin I/XRCC1), (2) regulate transcription (e.g., Mediator, TLE corepressor), (3) function as insulators (e.g., CTCF), and (4) methylate DNA (e.g., DNMT-1) (Figure 5) (Caiafa *et al.,* 2009; Caiafa and Zlatanova, 2009; El-Khamisy *et al.,* 2003; Guastafierro *et al.,* 2008; Hassa *et al.,* 2005; Heale *et al.,* 2006; Ju *et al.,* 2004; Malanga and Althaus, 2005; Pavri *et al.,* 2005; Pleschke *et al.,* 2000; Zampieri *et al.,* 2009). Many of these binding partners have been reported to be PARylated as targets of PARP-1 catalytic activity (Kim *et al.,* 2005; Kraus, 2008;

Kraus and Lis, 2003). Covalent attachment of PAR is thought to alter the activity of target proteins through both steric and charge effects, ultimately preventing protein-protein interactions, protein-nucleic acid interactions, enzymatic activity, or subcellular localization (Hassa and Hottiger, 2008; Schreiber *et al.*, 2006).

Known or suspected targets of PARP-1 catalytic activity include histones, transcription factors, nuclear enzymes, and nuclear structural proteins. For example, PARP-1 can PARylate histones, especially H1, H2A, and H2B, which may play a role in the regulation of chromatin structure, although the extent of histone modification and its relevance to nuclear processes remains to be clarified (D'Amours *et al.*, 1999; Kim *et al.*, 2005; Kraus, 2008; Kraus and Lis, 2003). PARP-1 also PARylates a number of DNA repair proteins, including p53 (Kanai *et al.*, 2007; Mendoza-Alvarez and Alvarez-Gonzalez, 2001), which is not surprising, given PARP-1's well-characterized role in DNA repair. Although the functional significance of p53 PARylation has been elusive, a recent study suggests that PARylation of p53 on specific sites (likely Glu 255, Asp 256, and Glu 268) can prevent p53 export from the nucleus by blocking its interaction with the nuclear export receptor Crm1 (Kanai *et al.*, 2007). PARP-1 has also been reported to PARylate and alter the function of numerous other transcription factors, including CTCF, AP-1, YY1, and NF-kB (Kraus, 2008), as well as nuclear enzymes, such as aurora B kinase (Monaco *et al.*, 2005), thereby inhibiting their function. As these examples suggest, PARylation of target proteins by PARP-1 plays a central role in determining cellular functions of PARP-1.

1.3.1.4 Posttranslational Modifications of PARP-1

Like other nuclear proteins that play key roles in regulatory processes, PARP-1 is subject to a variety of covalent posttranslational modifications as endpoints of cellular signaling pathways. These include PARylation, acetylation, phosphorylation, ubiquitylation, and SUMOylation; the latter two were more recently discovered and are less well characterized (Cohen-Armon *et al.*, 2007; Hassa *et al.*, 2005; Kauppinen *et al.*, 2006; Martin *et al.*, 2009; Messner *et al.*, 2009).

PARylation: PARP-1 is PARylated by itself, PARP-2, and possibly other PARPs. Automodification of PARP-1 (i.e., auto-PARylation) may occur as an extensive addition of ADP-ribose in chains >200 units in length or as a more modest addition of a single unit or chains up to 20 units in length (i.e., mono- or oligoPARylation, respectively) (D'Amours *et al.,* 1999; Mendoza-Alvarez and Alvarez-Gonzalez, 1999). Whether this occurs primarily in *cis* or *trans* (i.e., intra- or intermolecularly, respectively) has been debated in the literature but is typically considered intermolecular (Altmeyer *et al.,* 2009; Alvarez-Gonzalez and Mendoza-Alvarez, 1995; Mendoza-Alvarez and Alvarez-Gonzalez, 1993, 1999). Extensive auto-PARylation of PARP-1 (e.g., in response to DNA damage) inhibits its DNA-binding and catalytic activities (D'Amours *et al.,* 1999). Biochemical and cell-based assays have shown that activation and auto-PARylation of PARP-1 result in its release from chromatin (Kim *et al.,* 2004; Petesch and Lis, 2008; Tulin and Spradling, 2003; Wacker *et al.,* 2007). The effect of less-extensive auto-PARylation of PARP-1 is not clear; modestly modified PARP-1 may have altered activities but retain its association with chromatin.

Initial reports suggested that PARylation of PARP-1 occurred on as many as 28 glutamate residues, primarily in the AMD and DBD (D'Amours *et al.,* 1999; Schreiber *et al.,* 2006). In contrast, a recent study has shown that the glutamate residues in the AMD are not required for PARylation of PARP-1 (Altmeyer *et al.,* 2009). Rather, based on amino acid substitutions (i.e., Lys to Arg), the authors conclude that at least three lysine residues in the AMD (Lys 498, 521, and 524) are sites of automodification on PARP-1 (Altmeyer *et al.,* 2009).

Phosphorylation and Acetylation:

PARP-1 is phosphorylated by ERK1/2 at Ser 372 and Thr 373, and JNK1 at undetermined sites (Kauppinen *et al.,* 2006; Zhang *et al.,* 2007). The former is required for maximal PARP-1 activation after DNA damage (Kauppinen *et al.,* 2006), whereas the latter promotes sustained PARP-1 activation during H_2O_2-induced nonapoptotic cell death (Zhang *et al.,* 2007). A recent proteomic analysis has

identified additional phosphorylation sites in PARP-1, as well as sites in PARG, that will be good candidates for further functional analyses.

PARP-1 is acetylated by the acetyltransferases p300/CBP and PCAF (Hassa *et al.*, 2003, 2005; Rajamohan *et al.*, 2009). The acetylation of PARP-1 is reversed by a number of deacetylases, including Sirt1 (Hassa *et al.*, 2005; Rajamohan *et al.*, 2009). Acetylation of PARP-1 was first identified in the context of NF-kB dependent transcription, where it plays a critical role in regulating NF-kB target genes in immune cells (Hassa *et al.*, 2003, 2005). In cardiomyocytes, PARP-1 is acetylated as an endpoint of stress responses, resulting in the DNA damage-independent activation of PARP-1 (Rajamohan *et al.*, 2009). Acetylation of PARP-2 reduces its DNA-binding and enzymatic activities, and presumably the extent of auto-mono(ADP-ribosyl)ation (Haenni *et al.*, 2008).

Functional Interplay with Sirt1: Recent studies have begun to elucidate a functional interplay between PARP-1 and the NAD^+-dependent protein deacetylase Sirt1. Sirt1 is an important regulator of metabolism, cell differentiation and senescence, stress responses, and cancer through the regulation of chromatin structure and gene expression (Zhang and Kraus, 2009). PARP-1 and Sirt1 have been shown to function antagonistically; chemical activation of Sirt1 leads to reduced PARP-1 activity, and knockout of Sirt1 increases PARP-1 activity (Kolthur-Seetharam *et al.*, 2006). PARP-1 and Sirt1 are thought to compete for nuclear NAD^+, and a byproduct of the reactions they catalyze, nicotinamide, can inhibit both of their activities (Kim *et al.*, 2005; Zhang and Kraus, 2009). The interplay between PARP-1 and Sirt1, however, goes beyond simple competition for NAD^+. As noted above, acetylation of PARP-1 by PCAF is required for stress-induced cell death pathways. Deacetylation of PARP-1 by Sirt1 promotes cell survival (Rajamohan *et al.*, 2009). Unchecked PARP-1 activity in the absence of Sirt1 results in apoptosis inducing factor-mediated cell death. In mammalian cells, Sirt1 inhibits the expression of the PARP-1 gene, adding another layer of complexity to the functional interplay (Rajamohan *et al.*, 2009).Interactions and Functions of PARP-1 in the Nucleus: PARP-1 interacts with and PARylates proteins involved in DNA repair, transcription, DNA methylation, and regulation of

chromatin structure and histone modification to control physiological and pathological outcomes.

DNA Methylation:

Studies over the past decade have begun to link PARP-1-dependent PARylation with DNA methylation, a stable epigenetic mark that can be passed to daughter cells upon cell division and is associated with the repression of gene expression (Attwood *et al.*, 2002; Caiafa and Zampieri, 2005). One of the ways in which PARP-1 affects DNA methylation is by regulating the expression and activity of the DNA methyltransferase Dnmt1 (Caiafa *et al.*, 2009; Caiafa and Zlatanova, 2009). PARP-1 binds to the promoter of the Dnmt1 gene and protects it from DNA methylation-induced silencing in a PAR-dependent manner (Zampieri *et al.*, 2009).

SUMOylation and Ubiquitylation:

Recent studies have shown that PARP-1 is SUMOylated and ubiquitylated, modulating its role as a regulator of chromatin structure and transcription (Martin *et al.*, 2009; Messner *et al.*, 2009). PARP-1 interacts with and is SUMOylated by PIASy, a SUMO E3 ligase (Martin *et al.*, 2009; Stilmann *et al.*, 2009). PARP-1 contributes in many unique ways to the molecular biology of nuclear processes, playing key roles in the maintenance of genomic integrity, the regulation of chromatin structure and transcription, and the establishment of DNA methylation patterns, as well as a host of other processes (e.g., mitotic apparatus function, cell death pathways) (Fig. 3.1.1.2) (Hassa and Hottiger, 2008; Kim *et al.*, 2005). PIASy is recruited and released at the Hsp70 locus during the heat shock response with kinetics that mirror those of both PARP-1 and the SUMO-conjugating enzyme Ubc9 (Martin *et al.*, 2009). Interestingly, the SUMO-targeted ubiquitin ligase RNF4 poly ubiquitylates dPARP (*Droshophila* PARP) and presumably causes its clearance from the Hsp70 promoter via degradation (Martin *et al.*, 2009). These results fit well with the fact that dPARP regulates the chromatin structure at the Drosophila Hsp70 locus upon heat shock (Petesch and Lis, 2008; Tulin and Spradling, 2003).

1.3.1.5 PARP-1 and NAD^+ metabolism

As the ADP-ribose donor for PARP-1-catalyzed PARylation reactions, NAD^+ plays a central role in determining the function and activity of PARP-1. The synthesis of NAD^+ occurs in multiple cellular compartments, including the nucleus, which may be the most relevant source of NAD^+ for PARP-1 (Berger *et al.,* 2004; Rongvaux *et al.,* 2003). In mammals, NAD^+ is synthesized *de novo* from tryptophan, as well as through a salvage pathway leading from nicotinamide and catalyzed by the enzymes nicotinamide phosphoribosyl transferase (NAMPT) and nicotinamide mononucleotide adenylyltransferase (NMNAT; NMNAT-1 is the nuclear form) (Berger *et al.,* 2004; Rongvaux *et al.,* 2003) (Figure 4). Interestingly, nicotinamide is a natural endogenous inhibitor of PARP-1 (and Sirt1). Thus, the salvage pathway supports PARP-1 activity by depleting nicotinamide and producing NAD^+.

The enzymatic activities of PARP-1, NAMPT, and NMNAT are functionally linked. Stress-induced cell death due to PARP-1-dependent NAD^+ depletion in cardiomyocytes can be reversed by overexpression of NAMPT (Pillai *et al.,* 2005), supporting the conclusion that NAMPT catalyzes a rate-limiting step in NAD^+ synthesis (Revollo *et al.,* 2004). Furthermore, in addition to producing NAD^+ to support PARP-1 catalytic activity, NMNAT-1 also stimulates PARP-1 catalytic activity by binding to activated, automodified PARP-1 (Berger *et al.,* 2007). A recent study has shown that Sirt1 recruits NMNAT-1 to target gene promoters, presumably to supply NAD^+ for protein deacetylase reactions at the promoter (Zhang *et al.,* 2009). It is likely that a similar mechanism involving PARP-1 and NMNAT-1 supports PARylation of proteins at the promoters of PARP-1-regulated genes. As noted above, the enzymatic activities of PARP-1 and Sirt1 may also be linked through competition for limiting supplies of nuclear NAD^+ (Zhang *et al.,* 2009). Difficulty in accurately determining the concentrations of nuclear NAD^+, however, has hampered verification of this conclusion. Although functional interplay between PARP-1 and NAD^+-metabolizing enzymes in the nucleus has been established, the molecular mechanisms remain to be clarified.

1.3.1.7 PARP-1 overactivation depletes NAD^+

Based on what is known regarding bioenergetics, NAD^+ depletion causes ATP depletion; and the resulting drop in cellular energy leads to cell demise (D'Amours *et al.,* 1999; Chiarugi, 2005). NAD^+ is known to be a cofactor in several cellular metabolic processes needed for generating ATP such as glycolysis and the tricarboxylic acid cycle (D'Amours *et al.,* 1999). In addition, NAD^+ resynthesis requires at least 2-4 molecules of ATP while NAD^+ depletion blocks glyceraldehyde 3-dehydrogenase activity leading to the cell investing ATP in glycolysis, but without the return in NAD^+ (D'Amours *et al.,* 1999; Szabo and Dawson, 1998; Sheline and Wei, 2006). Thus, in support of the suicide hypothesis, PARP-1 activation leads to a block in the glycolysis. Indeed, replenishment of glycolytic and tricarboxylic acid cycle (TCA cycle) intermediates and substrates such as alpha-ketoglutarate or pyruvate, are neuroprotective (Chiarugi, 2005; Ying *et al.,* 2002). Also, administering NAD^+ to cells or overexpression of NAD^+ biosynthetic genes seem to rescue PARP-1 dependent cell death, suggesting that indeed, the NAD^+ decline associated with PARP-1 overactivation can cause cell demise (Chiarugi, 2005). However, NAD^+ replenishment probably prevents cell death through SIRT1 (Hassa *et al.,* 2006).

Recent findings suggest that the compartmentalization of NAD^+ within cells also must be considered when evaluating death induced by NAD^+ depletion. The mitochondrial pool of NAD^+ appears to be more relevant for cell death rather than the cytosolic and nuclear pools, as cell death is rescued upon preservation of NAD^+ in the mitochondria by cyclosporin A, or replenishment of NAD^+ by overepression of the biosynthetic nicotinic acid mononucleotide adenylyltransferase (NAMNT) (Alano *et al.,* 2007; Yang *et al.,* 2007). The drop in NAD^+ levels following PARP-1 overactivation reflects whole cell NAD^+. Thus, conclusions made from these studies need to be re-examined in light of the recent finding that the mitochondrial levels of NAD^+ remain at physiological levels following genotoxic stress and can support viability even when nuclear and cytoplasmic pools of NAD^+ are depleted (Yang *et al.,* 2007). Since overexpression of NAMNT leads to only a partial rescue of cell death (Alano *et al.,* 2007), thus PARP-1-dependent cell death may not exclusively depend on mitochondrial NAD^+ depletion. Reduction in cell death by cyclosporin A may also

not be attributed solely to NAD^+ preservation in the mitochondria, as it blocks mitochondrial permeability transition, which in itself is an important player in cell death signaling.

1.3.2 Poly(ADP-ribose) glycohydrolase (PARG)

PAR in some respects, resembles single-stranded nucleic acid polymers (D'Amours *et al.,* 1999). As described above, it functions as a covalent posttranslational modification, as well as a protein-binding matrix. Much of the focus on PAR to date has been on its synthesis and degradation, both of which occur on the timescale of minutes in the cell.

PAR is synthesized rapidly in response to a variety of physiological (e.g., hormone signaling) and stress-related (e.g., heat shock, DNA damage) stimuli (D'Amours *et al.,* 1999; Hakme *et al.,* 2008). As noted above, these stimuli ultimately result in the allosteric activation of PARP-1 catalytic activity, which in turn can lead to the auto-PARylation, as well as the transmodification of other protein targets. If extensive, auto-PARylation can inhibit PARP-1 enzymatic activity, which can block further PAR synthesis (D'Amours *et al.,* 1999; Hakme *et al.,* 2008). Very rapidly after synthesis (within seconds to minutes), PAR is degraded to ADP-ribose monomers, which may have signaling functions in the nucleus (Gagne *et al.,* 2006; Min and Wang, 2009). Structurally different types of PAR are degraded at different rates (i.e., short more rapidly than long, linear more rapidly than branched), which may influence their biological functions (Hassa and Hottiger, 2008).

Most PAR in the cell is degraded by the enzyme PARG, an enzyme with both exo- and endoglycosidase activities (actually a family of isoforms all encoded by the same gene) (Gagne *et al.,* 2006; Min and Wang, 2009) (Figure 4). In mice, targeted deletion of the 110 kDa PARG isoforms results in increased lethality in response to genotoxin exposure and septic shock relative to wild-type animals (Cortes *et al.,* 2004). Mice with complete deletion of all PARG isoforms are embryonic lethal. Trophoblast stem cells from these animals are viable only when cultured in the presence of a PARP

inhibitor, and they exhibit reduced growth, accumulation of PAR, and increased sensitivity to genotoxic stress (Koh *et al.,* 2004).

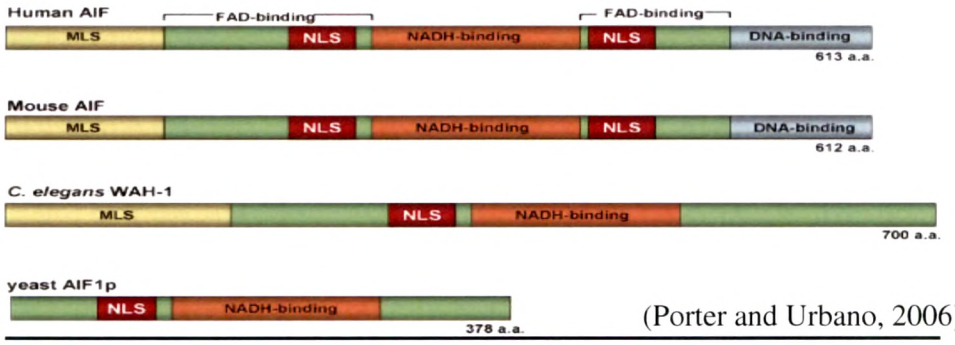
In Drosophila, increasing or decreasing dPARG levels phenocopies dPARP mutation, supporting a role for dPARG in removing PAR and, perhaps, facilitating multiple cycles of catalysis by individual PARP molecules (Tulin *et al.,* 2006). The available data highlight the importance of PAR catabolism for embryonic development, the maintenance of normal physiological states, and protection against genotoxic stress (Cortes *et al.,* 2004; Fisher *et al.,* 2007; Koh *et al.,* 2004).

Recently, the enzyme ADP-ribose-protein-hydrolase-3 (ARH3) was also shown to possesses intrinsic PARG activity (Oka *et al.,* 2006), suggesting that the mammalian genome may encode additional proteins with PARG activities. Other enzymatic activities, such as poly- and mono(ADP-ribosyl) protein hydrolase, as well as mono(ADP-ribosyl) protein lyase, may also act to remove PAR polymers and ADP-ribose monomers from target proteins (Hassa and Hottiger, 2008). In contrast to PARP, little is known about the role of PARG in cell function. There are, however, at least two mechanisms by which PARG could influence PARP-mediated cell death. First, PARG inhibition could slow the turnover of PAR and thereby limit NAD^+ depletion. Second, PARG inhibition could prevent the removal of PAR from PARP1. Because PARP1 is inhibited by extensive poly(ADP-ribosyl)ation, PARG inhibitors could thereby indirectly inhibit PARP1 activity. Report has shown that the PARG inhibitor gallotannin can markedly reduce death of astrocytes after oxidative stress (Alano *et al.,* 2004).

1.3.3 PARP-1 overactivation induces AIF (Apoptosis inducing factor) release

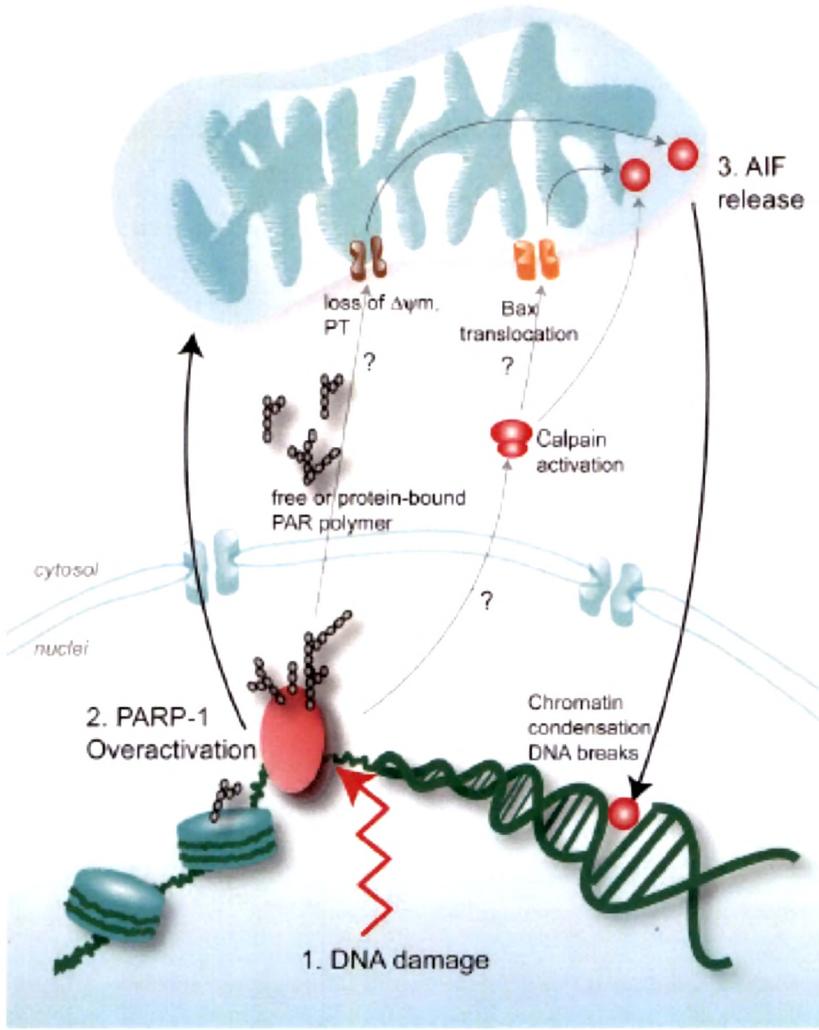
PARP-1 overactivation leading to AIF release from the mitochondria was first demonstrated by Yu *et al* (2002). In this study, PARP-1 KO mouse embryonic fibroblasts and neurons fail to release AIF. Furthermore, PARP-1 dependent cell death is reduced when AIF depleting antibodies are delivered into the cells. Most importantly, AIF translocation has been well-documented in several experimental

models of PARP-1 mediated cell death. AIF was purified from supernatants of mitochondria induced to undergo permeability transition (Susin *et al.,* 1996). This ubiquitously-expressed mitochondrial protein was isolated for its ability to translocate to nucleus and cause fragmentation of DNA into ~50-300kb pieces (Joza *et al.,* 2001). Its a 67-kDa protein that is believed to be converted to a 57-kDa protein upon cleavage of its putative mitochondrial localization sequence (Susin *et al.,* 1999). However, recent studies indicate that it is processed to a 62-kDa form upon cleavage of its mitochondrial localization sequence, and it is only processed to the 57- kDa form after a cell death stimulus (Otera *et al.,* 2005; Cao *et al.,* 2007) (Figure 1.13). Analysis of its sequence and its crystal structure reveal a glutathione reductase-like fold with an FAD-binding domain and an NADH binding domain as well as a C-terminal domain composed of five antiparallel beta-strands and two alphahelices (Mate *et al.,* 2002). The structure of the AIF protein reveals an overall positive electrostatic potential and homology to Bph4, a ferredoxin reductase in a dioxygenase from bacteria (Mate *et al.,* 2002). Analysis of recombinant AIF reveals NADH oxidase activity that can catalyze formation of superoxide anions (Vahsen *et al.,* 2004) further suggesting a role for AIF in redox processes within the mitochondria.



(Porter and Urbano, 2006)

Figure 1.12 Evolutionary conservation of functional domains in AIF family proteins The different functional domains of AIF or AIF-like homologues are shown color-coded for four species only. MLS, mitochondrial localization signal (yellow). Note that although yeast AIF-1 is located in mitochondria, a putative MLS has not been identified. NLS, nuclear localization signal (red); FAD, flavin adenine dinucleotidebinding regions (green); NADH-binding region (brown); DNA-binding region (blue) determines the pro-apoptotic function, which has been mapped for mammalian AIFs only. The NLS is always embedded in the FAD-binding domain. Yeast and *C. elegans* AIF homologues have only one putative NLS.



(David *et al.*, 2009)

Figure 1.13 PARP-1 overactivation leads to cell death. In the presence of death stimuli such as excessive DNA damage (1), PARP-1 overactivation (2) leads to the release of the death effector AIF from the mitochondria (3). The biochemical events mediating this nuclear-mitochondrial crosstalk are not completely known. Excess free or protein-bound complex PAR polymer may move from the nuclei to the cytosol where it disrupts protein-protein interactions. Since loss of mitochondrial membrane potential was observed in PARP-1 mediated cell death, PAR possibly binds cytosolic or mitochondrial proteins with roles in AIF release, mitochondrial embrane permeabilization or mitochondrial function. Other events downstream of PARP-1 overactivation include calpain activation, and Bax translocation to the mitochondria. These events appear to be important for AIF release: calpain is hypothesized to cleave AIF which is then released from mitochondria through pores formed by Bax.

The exact function of AIF in the mitochondria has been further clarified through the use of genetic knockdowns and conditional deletion of AIF. Harlequin (Hq) mutant mice contain a proviral insertion in the AIF gene that results in an 80% reduction of AIF protein levels (Joza *et al.,* 2001). Neonatal Hq mice exhibited 18% less respiratory chain complex I and 30% less catalase compared with WT mice (Zhu *et al.,* 2007). These mice develop a late-onset degeneration in the cerebellar granule cells and retinal ganglion cells (Joza *et al.,* 2005). In addition, there is marked oxidative stress in the brain and retina (Klein *et al.,* 2002), suggesting that AIF acts as a free radical scavenger in the mitochondria (Klein *et al.,* 2002). Later studies point that this increased levels of reactive oxygen radicals in Hq mice may be due to a role for AIF in the complex I activity of the electron transport chain (Vahsen *et al.,* 2004; Miramar *et al.,* 2001). This notion was confirmed by conditional deletion of AIF specifically in cardiac and skeletal muscle of mice, which resulted in impaired activity and decreased protein expression of mitochondrial complex I (Joza *et al.,* 2005). In addition to a role for AIF in redox processes, conditionally deleting AIF in embryonic telencephalon produced neurons with fragmented mitochondria, abnormal cristae structure and decreased survival (Cheung *et al.,* 2006).

Similar to cytochrome c, AIF assumes a deadly role once released from the mitochondria. Whereas cytochrome c is known to kill by the canonical apoptotic pathway of apoptosome formation (van Loo *et al.,* 2002), the mechanism by which AIF kills is not clear. Translocation of AIF to the nucleus induces chromatin condensation and DNA fragmentation possibly through cyclophilin A, even in the presence of caspase inhibitors (Loeffler *et al.,* 2001; Susin *et al.,* 1999; Joza *et al.,* 2001; Wang *et al.,* 2002). This nuclear apoptosis can be blocked by inhibition of cysteine proteases (Yuste *et al.,* 2005) and overexpression of hsp70 (Ravagnan *et al.,* 2001; Matsumori *et al.,* 2005). Translocation of AIF into the nucleus, and not its loss from the mitochondria, kills cells (Cheung *et al.,* 2006).

PAR is known to mediate cell death in part by inducing AIF release (Yu *et al.,* 2006). PAR releases AIF from the mitochondria *in vitro* when PAR generated from MNNG-treated nuclei is incubated with isolated mitochondria (Yu *et al.,* 2006). Moreover,

delivery of PAR into the cell induces AIF translocation and cell death (Andrabi *et al.*, 2006; Yu *et al.*, 2006). Cell death is mediated specifically by PAR because AIF release is abolished when PAR is catabolized by PARG or phosphodiesterase I (PDI) which degrade PAR polymers (Figure 1.14). In addition, overexpression of PARG prevents NMDA-induced AIF translocation (Andrabi *et al.*, 2006; Yu *et al.*, 2006).

Cd^{2+} is a toxic metal with no known biological function. It is increasingly important as an environmental hazard to both humans and wild life, and it exemplifies the double edge nature of many toxic substances. Cd^{2+} has been known to interefere in processes like cell death and tumorogenesis.

1.4 Molecular and cellular mechanisms of cadmium (Cd^{2+}) carcinogenesis

Cd^{2+} is an occupationally and environmentally important toxic element that is present in air, soil, sediment, and water. Non-occupational exposure is mainly from the diet and smoking (Satarug and Moore, 2004), with an estimated individual daily consumption of 30μg in the USA, and considerably higher in China and Japan (Joseph, 2009). Cd^{2+} accumulates in the human body with a long biological half-life of 2–3 decades. Its targets of toxicity include lung, liver, kidney, bone, cardiovascular system, and immune system (Fowler, 2009), where Cd^{2+}-induced cell death leads to loss of function. However, Cd^{2+} also acts as a cancer promoter through mitogenic effects on gene expression (reviewed in (Joseph, 2009, Beyersmann and Hechtenberg, 1997). It causes transformation in cultured cell lines (DiPaolo and Casto, 1979, Achanzar *et al.*, 2001) and produces malignant tumours in testes, prostate, lungs, pancreas and liver of experimental animals (Waalkes, 2003; Waalkes *et al.*, 1992). It is considered a human carcinogen by the International Agency for Research on Cancer (IARC) (International Angency for Research on Cancer) and occupational exposure has been associated with cancers of lung and possibly prostate, kidney, and pancreas (Waalkes, 2003). In addition to effects on gene expression and DNA repair, Cd^{2+} carcinogenesis probably involves inhibition of apoptosis (Joseph, 2009; Achanzar *et al.*, 2001; Achanzar 2002).

1.4.1 Cd^{2+} as a carcinogen

Cd^{2+} is a weak mutagen and a poor initiator of cancer (Waalkes, 2003). It interacts weakly with DNA and may act through epigenetic and (or) other mechanisms, including mitogenic effects on gene expression, inhibition of DNA repair, and inhibition of apoptosis (Waalkes, 2003), probably adaptive mechanisms in chronic low-dose exposure lead to lower ROS and apoptotic tolerance, thereby allowing proliferation of damaged cells with aberrant gene expression (Liu *et al.,* 2009). On the other hand, higher levels of Cd^{2+} that produce increased expression of genes of oxidative stress in rat lung epithelia are associated with apoptotic death of more than half the cells (Hart *et al.,* 1999).

Both oxidative stress and inhibition of repair of oxidative DNA damage undoubtedly play a role in Cd^{2+} carcinogenesis (Waisberg *et al.,* 2003; Liu *et al.,* 2009). Although Cd^{2+} does not redox cycle and therefore does not directly promote Fenton chemistry, it nevertheless increases cellular levels of reactive oxygen species (ROS) (Lieberthal *et al.,* 1998; Oh and Lim, 2006). Depletion of antioxidant defences might seem a plausible mechanism, but Cd^{2+} also facilitates adaptive increases in levels of glutathione, the Cd^{2+}-binding protein metallothionein, and catalase that are protective against peroxidative damage (Liu *et al.,* 2009; Lieberthal *et al.,* 1998; Oh and Lim, 2006; Templeton and Cherian, 1991; Chin and Templeton, 1993). Displacement of Fenton-active metals from other sites and inhibition of mitochondrial electron transport are also plausible mechanisms (Templeton and Cherian, 1991). Disruption of mitochondrial function itself may be a more important factor than ROS production in Cd^{2+}-induced cell death (Oh and Lim, 2006; López *et al.,* 2006). Cd^{2+}-induced oxidative damage to thiol groups of a number of cellular proteins can lead to denaturation and targeting for proteasomal degradation (Figueiredo-Pereira *et al.,* 1998).

1.4.2 Inhibition of DNA repair

Another possible mechanism of Cd^{2+} induced carcinogenesis is inhibition of repair pathways like base-excision repair, nucleotide-excision repair, recombinational repair

and mismatch repair. As to base-excision repair, low concentrations of Cd^{2+}, which do not generate oxidative base modifications as such, have been demonstrated to inhibit the repair of oxidative damage in mammalian cells (Dally and Hartwig, 1997). Regarding nucleotide-excision repair, Cd^{2+} inhibited the removal of thymine dimers generated by UV-irradiation by interfering with the first step of this repair pathway, i.e. the incision at the DNA lesion. Cd^{2+} at low concentrations inhibited the specific binding of repair proteins to damaged DNA. Furthermore, Cd^{2+} inhibited the bacterial repair enzyme formamido–pyrimidine–glycosylase and the specific DNA binding of the mammalian protein XPA that is essential for DNA damage recognition during nucleotide-excision repair (Asmuss *et al.*, 2000). Both proteins are members of the family of zinc-finger proteins, and the inhibitory effect of Cd^{2+} was assigned to a substitution of zinc by Cd^{2+} (Hartwig, 2001). Increased Cd^{2+} exposure were associated with reduced 8-oxoguanine repair and also inversely correlated with levels of DNA strand breaks in lymphocytes of Cd^{2+} exposed workers (Hengstler *et al.*, 2003). As to mismatch repair, Jin *et al* (2003) found that chronic exposure of yeast to environmentally relevant Cd^{2+} resulted in extreme hypermutability because Cd^{2+} reduced the capacity for mismatch repair, and in extracts of human cells Cd^{2+} inhibited at least one step leading to mismatch removal.

The elimination of a premutagenic modification of a nucleoside triphosphate precursor of DNA was also inhibited by Cd^{2+}. Oxidation of dGTP produces 8-oxo-dGTP which is misincorporated into DNA and causes AT→CG transversions. Cells are protected against 8-oxo-dGTP by antimutagenic 8-oxo-dGTPases. Both bacterial and human dGTPases were inhibited by Cd^{2+} in a dose-dependent manner, providing a further mechanism that may contribute to the mutagenic and carcinogenic potential of this metal (Bialkowski and Kasprzak, 1998). Because Cd^{2+} is only weakly mutagenic as such but inhibits the repair of DNA lesions formed spontaneously or caused by other agents, the generation of genetic instability seems to be a major mechanism contributing to the carcinogenicity of this metal.

1.4.3 Cd^{2+} and cell proliferation

A recent report had shown that effects of Cd^{2+} on signaling through Ca^{2+}, cAMP, NO, NF-kB, and developmental pathways such as Wnt signaling, in addition to kinases (Thévenod, 2009). Three major mitogen-activated protein kinases (MAPKs) exist in mammalian cells, namely Erk, Jnk, and p38 kinase (Chang *et al.,* 2001). In general, Erk is activated by growth factor receptors and stimulates cell proliferation, whereas Jnk and p38 are responsive to genotoxic agents and stresses (Tibbles and Woodgett, 1999; Garrington and Johnson, 1999; Chuang *et al.,* 2000; Wada and Penninger, 2004). Cd^{2+} has been shown to activate each of these pathway (Chuang *et al.,* 2000; Hung *et al.,* 1998), albeit with differential effects in different cell types. Cd^{2+} (1.5μM for up to 60 min) activated Erk1/2 and p38, but not Jnk, in chicken hepatoma cells (Elbirt *et al.,* 1998). Higher concentrations of Cd^{2+} (>100 μM for 3 h) persistently activated all three kinases in human lung carcinoma cells (Chuang *et al.,* 2000; Chuang and Yang, 2001), whereas at lower concentrations Erk activity was decreased and Jnk was only transiently increased, with no effect on p38 (Chuang and Yang, 2001). Activation of p38 was confirmed at 100μM Cd^{2+} in rat brain tumour cells, but was absent at 60μM, whereas the opposite was true of Erk1/2 (Hung *et al.,* 1998). In addition to activating the MAPKs, Cd^{2+} is also found to activate Ca^{2+}/calmodulin-dependent protein kinase-II (CaMK-II) in mouse mesangial cells (Liu and Templeton, 2008 and Liu and Templeton, 2007). The CaMKs are a family of broadspecificity kinases that serve as general integrators of Ca^{2+} signalling (Hook and Means, 2001; Hudmon and Schulman, 2002). They have been linked to oncogene induction in several cell lines (Misra *et al.,* 1994; Antoine *et al.,* 1996). A general mechanism by which Cd^{2+} may increase the activity of multiple kinases is by increasing ROS, which in turn oxidize thiol groups on kinase-regulating phosphatises. It also seems possible that Cd^{2+} inactivates multiple phosphatases by direct interaction with their thiol groups.

1.4.4 Inhibition of apoptosis

Cd^{2+} was found to suppress apoptosis induced by chromium in CHO cells (Shimada *et al.,* 1998). Evidence has been presented that Cd^{2+} inhibits caspase-3 in this context (Yuan *et al.,* 2000). However, the IC50 for caspase-3 inhibition by Cd^{2+} is 8.7μM in

intact CHO cells and significantly greater (31μM) in a cell-free system (Yuan *et al.*, 2000). Thus, even if caspase inhibition contributes to suppression of apoptosis by Cd^{2+} in intact cells, additional anti-apoptotic mechanisms must contribute.

Transformation of cells by Cd^{2+} may itself be associated with a decrease in apoptotic potential. When human prostatic epithelia are transformed by Cd^{2+}, they show a decreased apoptotic potential that may in part result from decreased caspase expression, and an increase in the ratio of Bcl-2/Bax due to a decrease in pro-apoptotic Bax and an increase in anti-apoptotic Bcl-2 expression (Achanzar *et al.*, 2002). In this model, Cd^{2+} may select for cells defective in apoptosis (Achanzar *et al.*, 2000). After implicating oxidative stress in Cd^{2+}-induced apoptosis in lung epithelia (Hart *et al.*, 1999). Hart *et al.* subsequently showed a decreased apoptotic response to oxidative stress in Cd^{2+}-adapted cells (Hart *et al.*, 1999).

Cd^{2+} (10μM, 8h) suppressed chromatin condensation, DNA laddering, and caspase-3 activation in response to both the extrinsic (TNF-alpha induced) and intrinsic stimuli (camptothecin) in mesangial cells (Gunawardana *et al.*, 2006). It also inhibited caspase-8 and -9 activities, decreased levels of tBid, and suppressed release of pro-apoptotic cytochrome c from mitochondria (Gunawardana *et al.*, 2006). These results suggest that under some circumstances Cd^{2+} may act as a general inhibitor of caspase activation, but may also have other anti-apoptotic effects through factors affecting mitochondrial stability.

1.4.5 Cd^{2+}-induced necrosis

Cd^{2+} may initiate necrotic death through multiple mechanisms, such as ROS production and depletion of antioxidant defences (leading to lipid peroxidation and membrane damage), and enzyme inhibition (contributing to loss of ATP production and ionic regulation of the intracellular environment). Acute, but not chronic, parenteral Cd^{2+} exposure causes hemorrhagic necrosis in rat testes and the testes of non-human primates. Acute oral administration causes necrosis of the gastric and intestinal mucosa. In general, concentrations of greater than 50μM Cd^{2+} cause

necrosis both *in vivo* and *in vitro*, while low concentrations cause apoptosis (Lee and Thévenod, 2008). In the present study also this dose dependent effect of Cd^{2+} has been observed on the unicellular eukaryote, *D. discoideum.*

As noted above, Cd^{2+} can induce proximal tubular necrosis in the kidney (Squibb and Fowler, 1984; Aughey *et al.,* 1984). However, direct evidence of Cd^{2+} induced renal cell necrosis is not abundant. Both acute and chronic effects of Cd^{2+} on the kidney in animal studies, and chronic effects in humans, are characterized by cell loss and dysfunction with inflammation leading to interstitial nephritis and fibrosis, and proximal tubular degeneration with chronic proteinuria and tubular dysfunction characteristic of Fanconi syndrome (Lee and Thévenod, 2008). In cultured mesangial cells, treatment with $Cd^{2+}Cl2$ in the range of 0.1–20μM for various periods of time and under various conditions leads to loss of viable cells almost exclusively by non-necrotic mechanisms (Liu and Templeton, 2008; Xiao *et al.,* 2009 ; Liu and Templeton, 2007).

1.4.6 Induction of apoptosis

Cd^{2+} has been shown to induce apoptosis (programmed cell death) in various organs of rats *in vivo* (Habeebu *et al.,* 1998; Xu *et al.,* 1996, 1999), and *in vitro* in several mammalian cell systems (El Azzouzi *et al.,* 1994; Hamada *et al.,* 1996; Fujimaki *et al.,* 2000; Wätjen *et al.,* 2002), and internucleosomal DNA fragmentation in isolated bovine liver nuclei (Lohmann and Beyersmann, 1993). Cd^{2+} seems to induce apoptosis by a mitochondria-dependent pathway because activation of caspase-9 was observed in Cd^{2+}-treated HL60 leukemia cells and C6 rat glioma cells (Wätjen *et al.,* 2001). At variance, Cd^{2+} has been shown to induce apoptosis in normal human lung cells by a mechanism that was mediated by mitochondria but independent of caspase activity (Shih *et al.,* 2003); a mechanism very similar to this has been observed in our study. Further evidence for a mediation by mitochondria is the protection against Cd^{2+}-induced apoptosis by the antiapoptotic protein Bcl-2 (Biagioli *et al.,* 2001; Kim *et al.,* 2000) and the induction of the proapoptotic protein Bax by Cd^{2+} in primary epithelial lung cells (Lag *et al.,* 2002).

After subcutaneous injection of Cd^{2+} in rats, programmed cell death was induced in testes but not prostate (Xu *et al.,* 1999). The induction of apoptosis in testes was negatively correlated with expression of p53. Since p53 is a tumor suppressor gene, the latter result is interpreted as a possible enhancement of the risk of Cd^{2+} tumorigenesis. In accordance with these findings, Meplan *et al* (1999) found that in human breast cancer MCF7 cells, Cd^{2+} inactivated the p53 protein by disrupting its native structure and inhibiting its DNA binding ability. In this system, Cd^{2+} down-regulated the transcriptional activation of a reporter gene coupled to the p53 promoter and impaired the induction of p53 in response to DNA damaging agents. In contrast to cells of the reproductive tract, alveolar cells from rat lungs exhibited increased p53 and Bax expression in response to Cd^{2+} (Lag *et al.,* 2002). At variance with this observation, some antioxidants (N-acetyl-l-cysteine or butylated hydroxyanisole) inhibited the induction of apoptosis by Cd^{2+} in human promonocytic U-937 cells (Galan *et al.,* 2001) and glutathione or catalase prevented Cd^{2+}-induced apoptosis in rat C6 glioma cells (Wätjen and Beyersmann, 2004). In human myeloid cells, catalase attenuated the generation of apoptosis by various antitumor drugs but potentiated the induction of apoptosis by heat-shock or Cd^{2+} (Sancho *et al.,* 2003), and depletion of catalase suppressed Cd^{2+}-elicited apoptosis in a human lung tumor cell line. These seemingly controversial results can be interpreted in terms of different interference of Cd^{2+} with cellular signalling pathways. Some evidence for the participation of oxidative stress in Cd^{2+}-induced apoptosis was also described by Hart *et al* (1999) who observed an increase in protein-bound glutathione in response to Cd^{2+} exposure of a rat lung epithelial cell line.

In all cell systems tested so far, Cd^{2+} induced cytoprotective proteins including metallothioneins, γ-glutamyl cysteine synthetase and glutathione-S-transferase (Hart *et al.,* 1999). Alveolar epithelial cells adapted to Cd^{2+} exhibited a decreased sensitivity to induction of apoptosis by hydrogen peroxide (Eneman *et al.,* 2000). In Chinese hamster ovary cells, Cd^{2+} even protected against programmed cell death that was triggered by the prooxidant metal ion chromate (Shimada *et al.,* 1998). Comparing various cell lines, a negative correlation was observed between

metallothionein induction and the extent of apoptosis induced by Cd^{2+} (Shimoda *et al.,* 2001) or etoposide (Shimoda *et al.,* 2003).

No uniform picture emerges with respect to the cellular signaling pathways involved in Cd^{2+}-induced apoptosis. In CL3 human lung cancer cells, Cd^{2+} activated the protein kinases ERK and p38-MAPK (Chao and Yang, 2001) and JNK. In these cells, inhibition of ERK enhanced the extent of apoptosis induced by Cd^{2+}, whereas inhibition of p38-MAPK decreased it, suggesting that ERK favors survival and p38-MAPK decreases genomic stability. In a human T lymphoblastoid cell line, Cd^{2+} activated the protein kinases ERK, p38-MAPK and JNK by phosphorylation (Iryo *et al.,* 2000). These authors found that inhibition of ERK, but not inhibition of p38-MAPK, suppressed Cd^{2+}-induced apoptosis. At variance, inhibition of p38-MAPK protected against Cd^{2+}-induced apoptosis in porcine kidney epithelial cells. Hence, depending on the cell system studied activation of protein kinases by Cd^{2+} may either favor or counteract programmed cell death.

The induction of apoptosis by Cd^{2+} is not necessarily protective against malignant transformation. Achanzar *et al* (2000) treated normal human prostate cells with Cd^{2+} and observed the induction of the proto-oncogenes c-jun and c-myc, and the tumor suppressor gene p53. Only a fraction of the cells was committed to apoptosis whereas 35% of the cells exhibited increased metallothionein and stayed viable, suggesting a selection of apoptosis-defective cells. Further evidence for an acquired apoptotic resistance of the Cd^{2+}-adapted fraction of prostate cells is indicated by the down-regulation of apoptotic caspases and the increased expression of the antiapoptotic protein Bcl-2 (Achanzar *et al.,* 2002). Taking into account the inhibition of repair of DNA damage by Cd^{2+}, Cd^{2+}-adaptation may inhibit apoptosis, allow the accumulation of critical mutations and favor the clonal expansion of pre-neoplastic cells towards tumor development (Hart *et al.,* 2001).

1.4.7 Reactive oxygen species and interference with the cellular antioxidant system

Various studies have shown that Cd^{2+} carcinogenicity seems to be crucially mediated by the production of reactive oxygen species. Cd^{2+} is known to induce the production of hydroxyl radicals, superoxide anions, nitric oxide and hydrogen peroxide (Galan *et al.*, 2001; Stohs *et al.*, 2001). Cd^{2+} also increases the levels of lipid peroxidation in liver (El-Maraghy *et al.*, 2001) and liver mitochondria of exposed rats (Casalino *et al.*, 1997) and in cultured rat hepatocytes (Muller, 1986). These findings reinforce the conclusions from the data on Cd^{2+}-induced ROS generation. The level of malondialdehyde is decreased if the exposure is maintained for 2 weeks (El-Maraghy *et al.*, 2001) suggesting an adaptative response.

Short-term exposure to Cd^{2+} has been shown to decrease the activities of almost all of these enzymes *in vitro* and *in vivo*, whereas with more elevated doses and extended exposure also enhancement of activities was found, probably because of adaptive induction of genes. Cd^{2+} can decrease the cellular glutathione content (which is known to scavenge intracellular reactive oxygen species by a direct reaction, or via the GSH peroxidase/GSH system), or the activities of superoxide dismutase (which catalyses the conversion of the superoxide anion radical to molecular oxygen and H_2O_2 and thus protects against superoxide-induced damage), glutathione peroxidase (which converts H_2O_2 and lipid peroxides to H2O and unreactive hydroxyl fatty acids, respectively) and catalase (which catalyses the conversion of H_2O_2 to water and molecular oxygen) (El-Maraghy *et al.*, 2001; Ochi *et al.*, 1987; Tatrai *et al.*, 2001). The decrease in the activity and/or intracellular levels of antioxidants caused by Cd^{2+}, together with the generation of radicals that are produced during normal metabolism, may explain the increase in lipid peroxidation and DNA damage in cells.

1.4.8 Gene regulation and signal transduction

Dysregulation of gene expression is regarded as a major factor in a multi-stage model of chemical carcinogenesis. Especially the induction of cellular proto-oncogenes (Hanahan and Weinberg, 2000) and stimulation of proliferation of committed cells

(Cohen, 1998) have been shown to play critical roles in the promotion stage after an initiating mutational event. On the other hand, induction of stress response genes is regarded as a protective mechanism providing detoxification both by heavy metal binding and generation of antioxidant substances. Implications in Cd^{2+} carcinogenesis and possible mechanisms involved are discussed below.

Immediate early response genes (IEGs)

Immediate early response genes (IEGs) are protooncogenes that undergo early transcriptional activation when quiescent cells are exposed to mitogenic substances such as Cd^{2+}. The IEGs most studied with respect to their involvement in the toxicity and carcinogenesis of Cd^{2+} are c-fos, c-jun and c-myc. Cd^{2+}-induced overexpression of these genes has been noticed in rat L6 myoblasts (Jin and Ringertz, 1990; Abshire *et al.,* 1996a,b), in rat kidney NRK-49F cells (Tang and Enger, 1993), in pig kidney LLC-PK1 cells (Matsuoka and Call, 1995), in rat and human mesangial cells (Templeton *et al.,* 1998; Wang and Templeton, 1998), in human prostate epithelial cells (Achanzar *et al.,* 2000) and in BALB/c-3T3 cells (Joseph *et al.,* 2001).

Metallothionein genes

Genes encoding for metallothionein (a low molecular weight protein containing about 30% cysteine) are the most studied genes with respect to the potential of Cd^{2+} to induce gene expression. Similar to the IEGs, subtoxic concentrations of this metal result in rapid and significant induction of MT *in vitro* and *in vivo*. Metallothioneins sequester Cd^{2+} with high affinity resulting in decreased availability of Cd^{2+} capable of interacting with cellular targets to elicit toxicity, including carcinogenicity. Lack of expression of MT protein, under basal and Cd^{2+}-stimulated conditions, has been regarded as one of the major underlying causes of tissue susceptibility to Cd^{2+} toxicity and/or carcinogenicity. An inverse relationship has in general been noticed between MT content and sensitivity of cultured cells and tissues of animals to Cd^{2+} (Waalkes *et al.,* 1992) and between basal or induced MT levels in human tumor cell lines and susceptibility to apoptosis (Shimoda *et al.,* 2003).

Heat-shock genes

Heat-shock proteins are cellular chaperones that are induced by hyperthermia and other environmental stresses such as exposure to toxic chemicals. Induction of HSPs

is generally considered as an adaptive response enabling cells to perform functions essential for survival under conditions of stress, including those induced by exposure to Cd^{2+}. Within the cells, Cd^{2+} induces the generation of denatured or abnormal proteins by reacting with vicinal thiol groups or by substituting for zinc in proteins; this has been recognized as the signal for the induction of HSPs (Parsell, 1994). Exposure of cells or whole animals to Cd^{2+} results in a significant induction of HSP10, HSP32, HSP40, HSP60, HSP70, HSP89, HSP90, and HSP110 genes. Induction of heat-shock genes in general occurs at Cd^{2+} concentrations more elevated than those effective in IEG induction, but HSP induction still may precede organ toxicity of this metal (Goering *et al.,* 1993).

Transcription factors

The potential of Cd^{2+} to influence the activity of several transcription factors leading to deregulation of gene expression has been demonstrated *in vitro*. The proto-oncogenes c-fos and c-jun code for proteins which are members of the AP-1 element that functions as a transcriptional factor regulating the expression of a large number of genes controlling cell growth and division (Angel and Karin, 1991). As described above, Cd^{2+} is a powerful inducer of c-fos and c-jun and this has been considered as a major mechanism for Cd^{2+}-induced cell transformation and tumorigenesis. Several other transcription factors, for example, metal regulatory transcription factor 1 (MTF1), upstream stimulator factor (USF), nuclear factor κB (NF-κB), and NF-E2-related factor (NRF2) are activated by exposure to Cd^{2+} resulting in modulation of gene expression (Alam *et al.,* 2000; Li *et al.,* 1998; Misra *et al.,* 2002; Smirnova *et al.,* 2000; Thevenod *et al.,* 2000). In contrast, exposure of cells to Cd^{2+} resulted in the suppression of the DNA binding activities of the transcription factors hypoxia-inducible factor-1 (HIF-1) (Chun *et al.,* 2000; Obara *et al.,* 2003) and Sp1. HIF-1 is involved in the control of expression of the erythropoietin gene, whereas Sp1 plays a key role in cell cycle proliferation and its inactivation leads to cell death.

Translation factors

Recently, the effect of Cd^{2+} on the expression of genes regulating translation has been reported (Joseph *et al.,* 2002). The whole process of protein synthesis or translation (initiation, elongation and termination of peptide chain synthesis) is regulated by the expression of several genes, collectively called translation factors. Two such genes,

translation initiation factor 3 (TIF3) and translation elongation factor-1δ (TEF-1δ), were found to be overexpressed in BALB/c-3T3 cells that were transformed by exposure to $Cd^{2+}Cl2$ (Joseph *et al.,* 2002). Cloning the $Cd^{2+}NAs$ for TIF3 and TEF-1δ and expression of the encoded proteins by transfection in mammalian cells resulted in transformation and tumorigenesis illustrating the oncogenic potential of both TIF3 and TEF-1δ. Furthermore, inhibition of the overexpressed TIF3 and TEF-1δ by employing the corresponding antisense mRNAs in the Cd^{2+}-transformed BALB/c-3T3 cells resulted in a significant reversal of the transformed phenotype of the cells. These results support the hypothesis that cell transformation and tumorigenesis induced by Cd^{2+} may be, at least in part, mediated through the overexpression of translation factors (Joseph *et al.,* 2002). Further experimental evidence, especially from animal experiments, is required to support this conclusion.

Miscellaneous genes

Recently developed techniques such as differential display and microarray analysis have facilitated the identification of a large number of genes exhibiting alterations in expression in response to exposure to Cd^{2+}. Liao and Freedman (1998) studied the ability of this metal to affect gene transcription in the nematode Caenorhabditis elegans by differential display analysis. Forty-nine $Cd^{2+}NAs$ whose steady-state levels of expression changed two to six-fold in response to Cd^{2+} exposure were identified. In addition to the genes involved in stress response, genes for collagen, rRNAs, pyruvate carboxylase, DNA gyrase, ß-adrenergic receptor kinase, human hypothetical protein KIAA0174, for several novel proteins were identified.

1.4.9 Mechanisms of Cd^{2+}-induced alterations in gene expression

The actual mechanisms responsible for Cd^{2+} induced deregulation of gene expression are better understood now than in the recent past. Several possible mechanisms, including effects on secondary messengers such as reactive oxygen species (ROS) and intracellular Ca^{2+}, transcription factors, cellular signal transduction cascades involving kinases, and DNA–cytosine methylation are considered to be responsible for the Cd^{2+}-induced deregulation of gene expression.

1.5 *Dictyostelium discoideum*

D. discoideum often referred to as "slime mold" or "social amoeba", is one of the simplest studied eukaryotes that possesses true multicellularity (Raper, 1984). The cellular slime molds were formerly considered to be 'lower fungi.' Although they superficially resemble fungi in certain respect they are included in the Kingdom Protista. Individual cells resemble small amoebae and move and feed in an amoeboid manner, thus they are called 'myxamoebae' (to distinguish them from true amoebae). *D. discoideum* was first discovered in 1935 in a forest in North Carolina and has since been found, along with similar genera, in many such environments around the world. *D. discoideum* can be found in soil and moist litter leaves. The primary diet of *D. discoideum* consists of bacteria such as *Klebsiella, E.coli* etc. that are found in soil. These bacteria secrete folic acid which attracts amoebae. Under good nutritive conditions amoebae remain unicellular and grow as primitive animal like cells either on bacteria (wild type) or in a semi defined growth medium used for the axenic laboratory strains.

D. discoideum development

D. discoideum has an intriguing way of becoming multicellular, under nutrient depleted conditions. This represents a novel developmental transition which is absent in most other multicellular lineages. The process involves the aggregation of individual cells followed by well orchestrated movements to spatially organize the cell types. Starvation initiates the creation of biochemical machinery which includes glycoproteins and adenylyl cyclases (Gilbert, 2006). The glycoproteins allow for cell-cell adhesion and adenylyl cyclases synthesize cyclic AMP. Cyclic AMP is secreted by the amoebae to attract neighboring cells to a central location. As they move towards the signal, they bump into each other and stick together by the use of glycoprotein adhesion molecules.

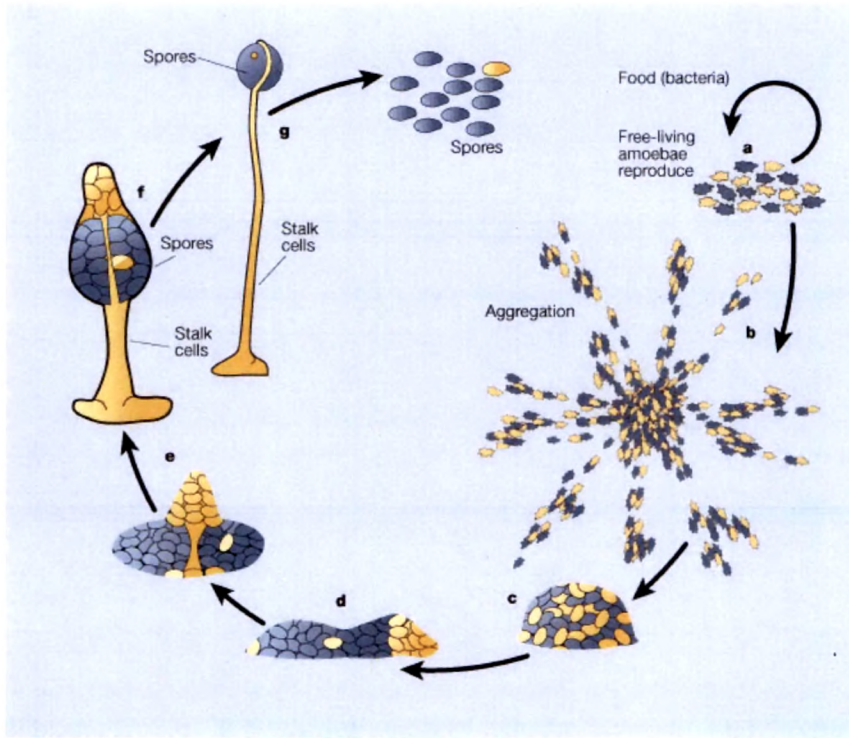
Starving cells stream together by chemotaxis towards autocrine signals and form aggregates that contain 10^5 cells, to form a multicellular mass, the mound (tight

aggregate). The driving force behind this process is chemotaxis towards a pulsatile source of extracellular cAMP (Roos *et al.*, 1975). When cells form an aggregate, cAMP concentration is thought to rise to the micromolar range (Abe *et al.*, 1983). During aggregation, oscillatory waves of cAMP are generated from the center of the aggregating territory and are propagated toward neighboring cells. Initially, amoebae move as individual cells towards the signal, however, as they reach near the source and cell density increases, cells coalesce into multicellular streams.

A transcriptional cascade is activated, leading to the emergence of different cell types that self organize within the aggregate (Firtel, 1995; Kimmel and Firtel, 1991; Loomis, 1996). After about 6-8 hours of starvation, a flat loose aggregate is formed with indistinct borders. A sheath of mucopolysaccharide and cellulose is laid on a group of cells to form a tight aggregate or mound (Wilkins and Williams, 1995). During development, 20% cells differentiate into prestalk and the remaining 80% differentiate to form prespore cell types, in specified ways, and form a slug. The relative proportion of prestalk cells within slugs varies between 10% to 30% depending on the slug size and shape (Rafols *et al.*, 2001). Differentiation inducing factor (DIF) induces stalk cell differentiation in *D. discoideum* and acts as the morphogen in the generation of the prestalk/prespore pattern during development (Masento *et al.*, 1988). The slug undergoes transient or prolonged migration depending on the environmental conditions. Recent work has focused that spatial gradients of DIF does not act as the primary signal for cell type choice (Thompson and Kay, 2000). Rather, the choice of cell type appears to rest on a basis that is quite the opposite of morphogen dependent spontaneous patterning, namely the existence of functional differences in the form of preexisting heterogeneities, between the members of an apparently homogeneous cell mass.

Fate of the cells is predetermined during unicellular form only. Pre aggregation amoebae can differ in many ways, which include nutritional status, cell size, cell cycle phase at starvation, cellular calcium content (Nanjundiah, 1997) and sensitivity to DIF-1. The cell cycle phase at starvation has also been implicated in

determining the fate i.e. amoebae in S and early G2 phases at starvation exhibit a prestalk tendency (Weijer *et al.,* 1984, MCd^{2+}onald and Durston, 1984, Gomer and Firtel, 1987). Thus Calcium concentration and the cell cycle phase at the time of starvation decide the cell fate. High (Ca^{2+}) levels during S-phase is not required for cell cycle progression but for cell type choice mechanism at the onset of starvation, and these cells tend to follow the prestalk pathway while cells with low Ca^{2+} levels tend to form prespore (Azhar *et al.,* 1997; Saran, 1999). Cell fate in *D. discoideum* is thus decided based on intercellular heterogeneity as the primary factor behind cell fate choice.



(1.bp.blogspot.com/.../s1600/slime^{+}mold^{+}03.jpg)

Figure 1.14 Life cycle of *D. Discoideum*

After a variable period of migration, the slug settles at one place and cells near the tip form a sheath within which the cells expand and vacuolize to form the stalk, and then extends vertically. The posterior end spreads out with the anterior end raised in the air, forming what is called the "Mexican hat," and the culmination stage begins. The

prestalk cells and prespore cells switch positions in the culmination stage in order to form the mature fruiting body. The anterior end of the Mexican hat forms a cellulose tube, which allows the more posterior cells to move outside of the tube to the top, and the prestalk cells move down. This rearrangement forms the stalk of the fruiting body made up of the cells from the anterior end of the slug, and the cells from the posterior end of the slug are on the top and now form the spores of the fruiting body. Thus spore is supported by a skeleton of dead cells that are arranged as a stalk and a basal disc, which anchors the stalk to the substratum.

When the spores are dispersed, under favorable conditions, they germinate by splitting the spore case longitudinally and escaping as small but normal amoebae. This complex series of stages give a two fold selective advantage to the organism i.e. to permit the dispersal of cells from an area in which they are starving and to provide a dormant stage to resist unfavorable conditions.

Molecular aspects of *D. discoideum* development

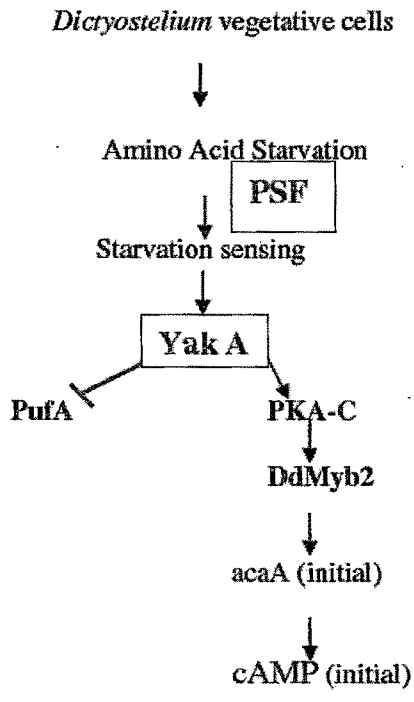
The process of aggregation bridges the feeding unicellular form of *D. discoideum* to a starving multicellular form. Many biosynthetic genes expressed at growth stage are downregulated and genes involved in development are upregulated (Mir *et al.,* 2007). Amino acid starvation represses the development of these amoebae (Marin, 1976). Recent investigations have revealed several components involved in regulating the initiation of development (Souza *et al.,* 1999; Kon *et al.,* 2000; Zeng *et al.,* 2000), however little information exists on how the cells exactly sense starvation and in particular amino acid deprivation. Studies have now implicated the Target of Rapamycin (TOR) pathway in the process of sensing these two nutrients (Lee *et al.,* 2005).

Cell cycle arrest

Upon amino acid depletion, *D. discoideum* cells undergo cell cycle arrest. YakA, a serine/threonine protein kinase governs this transition by regulating the cell cycle, repressing growth phase genes and inducing developmental genes. *yakA* is induced by

starvation and its expression is controlled by an extracellular prestarvation factor (PSF) that accumulates during vegetative growth.

YakA mediates the initiation of development by repressing *pufA* expression. PufA, a translational regulator inhibits catalytic subunit of cAMP-dependent protein kinase A (PKA-C) translation by binding to a region at the 3'end of the *PKA-C* mRNA (Souza *et al.,* 1999). Thus, YakA acts as a regulator switch between vegetative and developmental gene expression by relieving the negative control on PKA-C expression, which in turn activates DdMyb2 transcription factor and all further downstream events which include activation of adenylyl cyclase leading to production of the differentiation inducing signal cAMP. The adenylyl cyclase gene *aca*A is one of the first genes expressed upon starvation (Fig. 1.23). ACA produces extracellular cAMP that induces chemotaxis and aggregation in neighboring cells. Intercellular signaling by secreted cAMP then induces the expression of another set of genes for further stages of development. Therefore the components that mediate the induction of adenylyl cyclase have the central role in the growth/development transition in *D. discoideum.*



(Mir *et al.*, 2007)

Figure 1.15 Signaling during initiation of *D. discoideum* development

D. discoideum and Programmed Cell Death

Cell death occurs in many places along the phylogenetic tree other than in animals. Investigation of cell death in other organisms may reveal phenomenological convergence or molecular conservation and then yield invaluable comparative information (Golstein, 1998). The slime mold *D. discoideum* is an early conditional multicellular organism that shows developmental cell death. It has been observed by many workers that *D. discoideum* demonstrates cell death mechanism similar to that seen in some of the higher eukaryotes. Another ontogenic reason being the relatively simple pattern of development in this organism facilitates the study of cell death that occurs during development. There are methods that allow triggering *in vitro* differentiation without morphogenesis and thus facilitate the isolation of dying cells for study (Kay *et al.*, 1987).

D. discoideum during starvation induced developmental process exhibits PCD in the 20% stalk cell population. *D. discoideum* exhibits caspase independent type of cell death (paraptosis) during its development which occurs even in the presence of caspase inhibitors. Paraptosis is characterized by the absence of oligonucleosomal DNA fragmentation. Developmental cell death requires starvation and presence of DIF (Cornillon *et al.*, 1994). *D. discoideum* cells in conditioned medium undergo cell death that shares essential features with mammalian cell apoptosis. This involves a loss of mitochondrial membrane potential (Ψm), resulting in the release of AIF from the mitochondria (Arnoult *et al.*, 2001). Stationary phase cells also exhibit similar kind of features (Tatischef *et al.*, 2001). It has been documented that *D. discoideum* undergoes an 'apparent' caspase independent programmed cell death (Olie *et al.*, 1998). The stalk cells show massive vacuolization, prominent cytoplasmic condensation and focal chromatin condensation (Olie *et al.*, 1998). The *D. discoideum* vacuolar cell death pathway does not require cellulose synthesis and includes early actin rearrangements (F-actin segregation, then depolymerization); contemporary with irreversibility, corresponding to the emergence and demise of highly polarized paddle cells (Levraud *et al.*, 2003). Contradictory observations have been made for the cell death in stalk cell in the presence of caspase inhibitors. Simbulan *et al.*, (1999) showed no effect on stalk cell death while on the other hand though caspase inhibitors

did not inhibit cell death they were observed to impair development in *D. discoideum*. Also these inhibitors show dose dependent increase in percent of stalkless fruiting bodies (Olie *et al.,* 1998).

According to Kawli *et al.,* (2002) there is no internucleosomal cleavage of DNA in *D. discoideum*. However, nuclear condensation and peripheralization does occur in stalk cells. It was also shown that the fraction of cells showing caspase 3 like activity increases and reaches a maximum of around 25 % in the slug stage correlating with proportion of stalk cells (Kawli *et al.,* 2002). Thus, cell death in *D. discoideum* shows some, but not all, features of apoptotic cell death as recognized in other multicellular systems. The molecular mechanism underlying this kind of cell death is yet to be understood (Kawli *et al.,* 2002).

Blast search results suggested that *D. discoideum* has a paracaspase gene, no metacasapase and a caspase gene. The paracaspase null mutants showed undiminished cell death *in vivo* and *in vitro*, in addition paracaspase inactivation led to no alteration in development. Thus programmed cell death does not require paracaspase (Bouffay *et al.,* 2004). As *D. discoideum* shares ancestry in some of the molecular mechanisms of cell death with mammalian cells and thus is a good model system to characterize paraptotic cell death. Thus the social amoeba *D. discoideum*, a powerful paradigm provides clear insight into the regulation of growth and development. In view to this *D. discoideum* is an excellent model system to study the role of PARP and PARG in caspase independent cell death. *D. discoideum* has potential PARP genes (Otto *et al.,* 2005). Our lab has established the role of PARP in *D. discoideum* development (Rajawat *et al.,* 2011).

Previous work done in the lab

Our lab studies describe the events during oxidative stress induced PARP mediated cell death in *D. discoideum* when the action of PARP is intercepted by PARP inhibitor benzamide as well as by PARP antisense (Rajawat, 2010). Our lab is first to report the involvement of PARP and the downstream events during oxidative stress

induced cell death and development in *D. discoideum*. It was demonstrated that oxidative stress induced cell death is also partially rescued by PARG inhibition. We put forth that *D. discoideum* exhibits paraptosis which is mediated by PARP. PARP and AIF are the major players governing *D. discoideum* cell death kinetics during paraptosis and necrosis induced by oxidative stress ((Rajawat, 2010; Mir, 2011).

Similarly, cell death induced by different stresses such as UV-C, staurosporine (STS), starvation and the interception by benzamide, a PARP inhibitor were also studied. STS induced paraptosis is also characterized by AIF mediated DNA fragmentation however, it was not affected with PARP inhibition. Thus PARP is a dispensable player of paraptosis (Mir, 2011).

Besides cell death developmental studies were also carried out. Constitutive down-regulation of PARP resulted in blocked development at slug formation while no effect was observed on growth. Studies on long term effects of PARP inhibition on *D. discoideum* development under oxidative stress demonstrated that second generation cells showed normal development signifying that PARP inhibition has no deleterious effect on *D. discoideum* development (Rajawat *et al.*, 2007; 2011). Based on these results our lab has proposed that presence of PARP is essential for complex differentiation and its function may be linked to multicellularity.

D. discoideum shows differential effects of oxidative and UV-C stress on development and spore germination as oxidative stress induced changes could be rescued by PARP inhibition, however many of the UV-C induced changes were not affected by PARP inhibition. Thus *D. discoideum* exhibits differential behavior to different stresses. In current study we are interested to observe the response of *D. discoideum* to Cd^{2+} stress and to find the link for nuclear-mitochondrial cross talk during PARP mediated cell death.

Thus the objectives of this study were,

1) Mechanism of Cadmium induced cell death in *Dictyostelium discoideum*

2) The role of poly (ADP-ribose) polymerase during Cadmium induced cell death

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