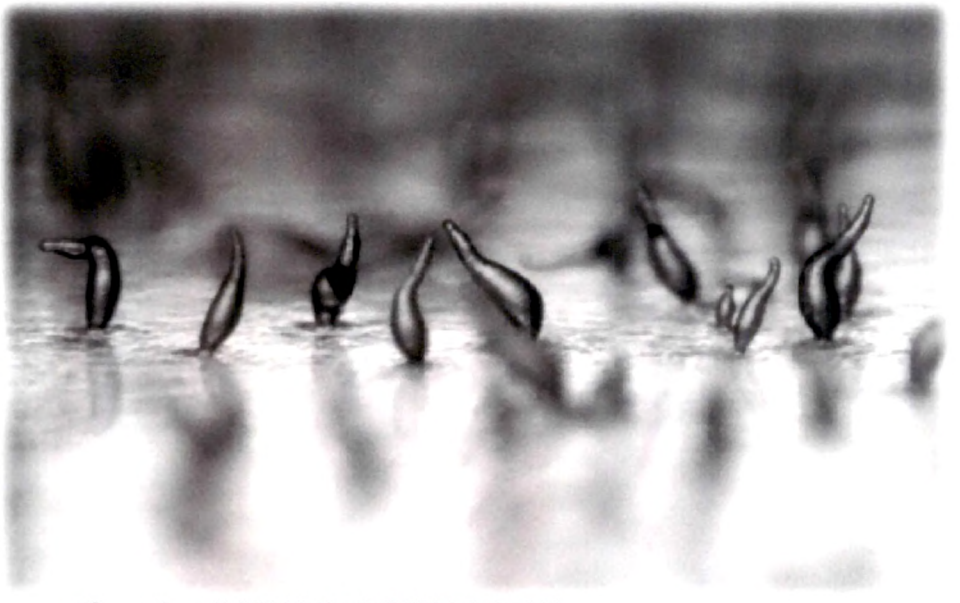
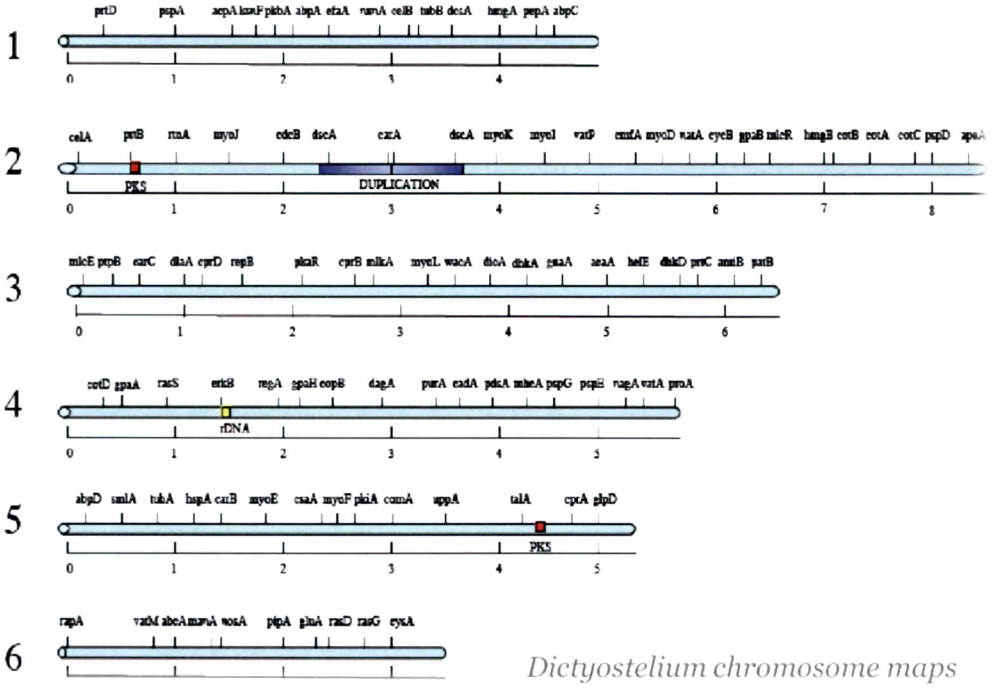
Death becomes exciting sometimes!!.....
.....discover how!





Dictyostelium chromosome maps

Introduction

'While I thought that I was learning how to live, I have been learning how to die.'

- Leonardo Da Vinci

1.1. Cell death

Life requires death however spiritual this might sound, from a scientific point of view this is the truth of life. Multicellular organisms are highly organized and conserved. They require crucial processes to sustain life and cell death follows similar characteristics from worms to mammals. Death is the inevitable fate for all living things including individual cells. Cell death is a state where biological function of living cell ceases. However, untimely death results in dysfunction and disease. Cell death is broadly classified into two types: necrosis and programmed cell death.

1.1.1. Necrosis

Necrosis is the premature death of cells and living tissues. It is an unordered and accidental form of cell death. Cellular necrosis can be induced by a number of sources, including injury, infection, cancer, infarction, poisons, and inflammation. Necrosis results from the additive effect of a number of independent biochemical events that are activated by severe depletion of cellular energy stores and thus is a passive phenomenon (Kanduc *et al.*, 2002). Necrosis typically begins with cell swelling, chromatin degradation, and disruption of the plasma membrane as well as organelle membranes. Late necrosis is characterized by extensive DNA fragmentation, organelle breakdown, and cell lysis. The release of intracellular content after plasma membrane rupture is the cause of inflammation in necrosis.

1.1.2. Programmed cell death

Programmed cell death (PCD) is death of a cell in any form mediated by an intracellular program. PCD is an important mechanism in both development and homeostasis in multicellular organisms for the removal of either superfluous, infected, transformed or damaged cells by activation of an intrinsic suicide program. It is a mechanism that enables metazoans to eliminate cells that threaten the animal's survival. PCD is carried out in a regulated process which generally confers advantage during an organism's life cycle. PCD serves fundamental functions during plant and metazoan (multicellular animals) tissue development. Programmed cell death, a central mechanism controlling multicellular development, leads to deletion of entire structures (e.g., the tail

in developing human embryos), sculpts specific tissues by ablating fields of cells (e.g., tissue between developing digits), and regulates the number of neurons in the nervous system. The term "programmed cell death" was coined by Lockshin and Williams in 1964 in relation to insect tissue development.

Types of PCD:

Type I cell death or Apoptosis

Type II cell death or Autophagy

Type III cell death or Nonapoptotic PCD

1.1.2.1. Type I cell death or Apoptosis:

In Greek, apoptosis translates to "dropping off" of petals or leaves from plants or trees. A German scientist Carl Vogt was first to describe the phenomenon of apoptosis in 1842. Professor James Cormack of the University of Aberdeen suggested the term "apoptosis". Kerr, Wyllie and Currie resurrected this term in 1972.

Apoptosis consists of a cascade of events leading to the ordered dismantling of cells. Apoptosis is an essential part of life for any multicellular organism and the manner in which most cells die is conserved. Apoptosis occurs normally during development and aging as a homeostatic mechanism to maintain cell populations in tissues. Apoptosis also occurs as a defense mechanism in immune reactions or when cells are damaged by disease or noxious agents (Norbury and Hickson, 2001). It may take place when a cell is damaged beyond repair, infected with a virus, or undergoing stressful conditions such as starvation. Apoptosis is characterized by maintenance of intact cell membranes during the suicide process so that the dying cell does not release its contents and trigger an inflammatory reaction in vicinity and also allows adjacent cells to engulf it. Cells undergoing apoptosis usually exhibit a characteristic morphology, including fragmentation of the cell into membrane bound apoptotic bodies, nuclear and cytoplasmic condensation and cleavage of the DNA into small oligonucleosomal fragments (Steller, 1995). The cells or fragments are then phagocytosed by macrophages (Fig. 1.1).

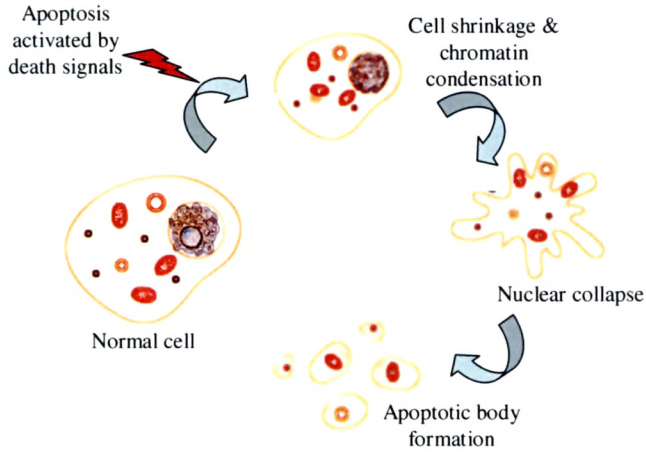


Figure 1.1: Apoptosis – the programmed death of a cell

The process of apoptosis is controlled by a diverse range of signals, which may originate either extracellularly (extrinsic signals) or intracellularly (intrinsic signals). Extracellular signals include toxins (Popov *et al.,* 2002), hormones, growth factors, nitric oxide (Brune, 2003) or cytokines, and therefore must either cross the plasma membrane or transduce to exhibit a response. These signals may positively (i.e., trigger) or negatively (i.e., inhibit) affect apoptosis. Signals that can trigger apoptosis include damage due to ionizing radiation, viral infection, extracellular signals and the same signals may promote survival in one cell type and evoke the suicide program in others (Steller, 1995). This suicide program sometimes involves the synthesis of specific messenger RNA molecules and their translation, and in such instances PCD can be suppressed by inhibiting transcription or translation (Steller, 1995).

A cell initiates intracellular apoptotic signaling in response to a stress, which may bring about cell suicide. Heat, radiation, nutrient deprivation, viral infection, hypoxia (Cotran *et al.,* 1998) and increased intracellular calcium concentration (Mattson and Chan, 2003) can all trigger the release of intracellular apoptotic signals in a damaged cell.

Many pathways and signals lead to apoptosis, but all these converge to common mechanisms that actually cause the death of a cell. After a cell receives stimulus, it undergoes organized degradation of cellular organelles by activated proteolytic caspases. Caspases are the central executioners of apoptosis. The term caspase denotes two key

characteristics: (i) they are cysteine proteases and use cysteine as the nucleophilic group for cleavage of the substrate and (ii) they are aspases and cleave the peptide bond C-terminal to aspartic acid residues (Alnemri *et al.*, 1996). Caspases involved in apoptosis are generally divided into two categories: The **initiator caspases** are characterized by an extended N-terminal prodomains, which include caspase - 2, -8, -9, -10. The **effector caspases** have smaller N-terminal prodomains and include caspase -3, -6, -7. All caspases are present in the cytosol as zymogens and must undergo proteolytic activation during apoptosis.

Mechanism for activation of caspases –

Mechanisms of caspase activation include (a) proteolytic cleavage by an upstream caspase, (b) induced proximity and (c) holoenzyme formation. Proteolytic cleavage by an upstream caspase is straight forward and effective, and is used mostly for activation of downstream effector caspases.

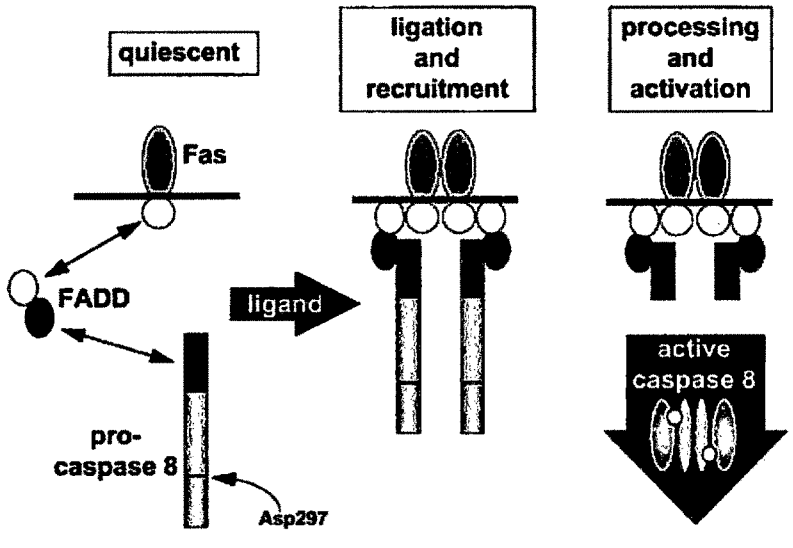


Figure 1.2: Model for caspase-8 activation

In the second mechanism, recruitment or aggregation of multiple procaspase-8 molecules into close proximity results in cross activation (Fig. 1.2). The actual process is most probably more sophisticated and more tightly regulated. In holoenzyme formation, cytochrome c and ATP dependent oligomerization of Apaf-1 allow recruitment of

procaspase-9 into the apoptosome complex. Activation of caspase-9 is mediated by means of proteolytic processing.

Activation of caspases does not result in the indiscriminate degradation of cellular proteins. Rather, caspases selectively cleave a restricted set of target proteins, usually at one or a few positions in the primary sequence (always after an aspartate residue). Proteolytic cleavage by caspases can lead to diverse results, depending on the nature of the substrate and the exact position of the cleavage site in the primary sequence. (Hengartner, 2002).

A cell undergoing apoptosis shows a characteristic morphology:

1. Cell shrinkage and rounding are shown because of the breakdown of the proteinaceous cytoskeleton by caspases.
2. The cytoplasm appears dense, and the organelles appear tightly packed.
3. Chromatin undergoes condensation into compact patches against the nuclear envelope in a process known as pyknosis, a hallmark of apoptosis (Susin *et al.*, 2000; Kihlmark *et al.*, 2001).
4. The nuclear envelope becomes discontinuous and the DNA is fragmented in a process referred to as karyorrhexis. The DNA breaks into several discrete chromatin bodies or oligonucleosomal units (Nagata, 2000).
5. The cell membrane shows irregular buds known as blebs.
6. The cell breaks apart into several vesicles called apoptotic bodies, which are then phagocytosed.

There are two apoptotic pathways i.e., Extrinsic and Intrinsic.

Extrinsic Pathway:

The extrinsic signaling pathways that initiate apoptosis involve transmembrane receptor mediated interactions. These involve death receptors that are members of the tumor necrosis factor (TNF) receptor gene superfamily (Locksley *et al.*, 2001). Members of the TNF receptor family share similar cyteine rich extracellular domains and have a cytoplasmic domain of about 80 amino acids called the "death domain" (Ashkenazi and Dixit, 1998). This death domain plays a critical role in transmitting the death signal from the cell surface to the intracellular signaling pathways. To date, the best characterized ligands and corresponding death receptors include FasL/FasR, TNF-α/TNFR1, Apo3L/DR3, Apo2L/DR4 and Apo2L/DR5 (Peter and Kramer, 1998; Suliman *et al.*, 2001; Rubio-Moscardo *et al.*, 2005). Upon ligand binding, cytplasmic adapter proteins are recruited which exhibit corresponding death domains that bind with the receptors. The binding of Fas ligand to Fas receptor results in binding of the adapter protein FADD, and the binding of TNF ligand to TNF receptor results in binding of the adapter protein TRADD with recruitment of FADD and RIP (Hsu *et al.*, 1995; Grimm *et al.*, 1996; Wajant, 2002). This signaling results into activation of procaspases via death receptor mediated pathway. Procaspases have prodomains which contain death effector domain (DED) (Cohen, 1997; Wang *et al.*, 2005). FADD then associates with procaspase-8 via dimerization of the death effector domain. At this point, a death inducing signaling complex (DISC) is formed, resulting in the autocatalytic activation of procaspase-8 (Kischkel *et al.*, 1995). Once caspase-8 is activated, executor caspases are activated (Fig. 1.3). Death receptor mediated apoptosis can be inhibited by a protein called c-FLIP which will bind to FADD and caspase-8, rendering them ineffective (Kataoka *et al.*, 1998; Scaffidi, 1999). Another point of potential apoptosis regulation involves a protein called Toso, which has been shown to block Fas-induced apoptosis in T cells via inhibition of caspase-8 processing (Hitoshi *et al.*, 1998).

Intrinsic 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 (Fig. 1.3).

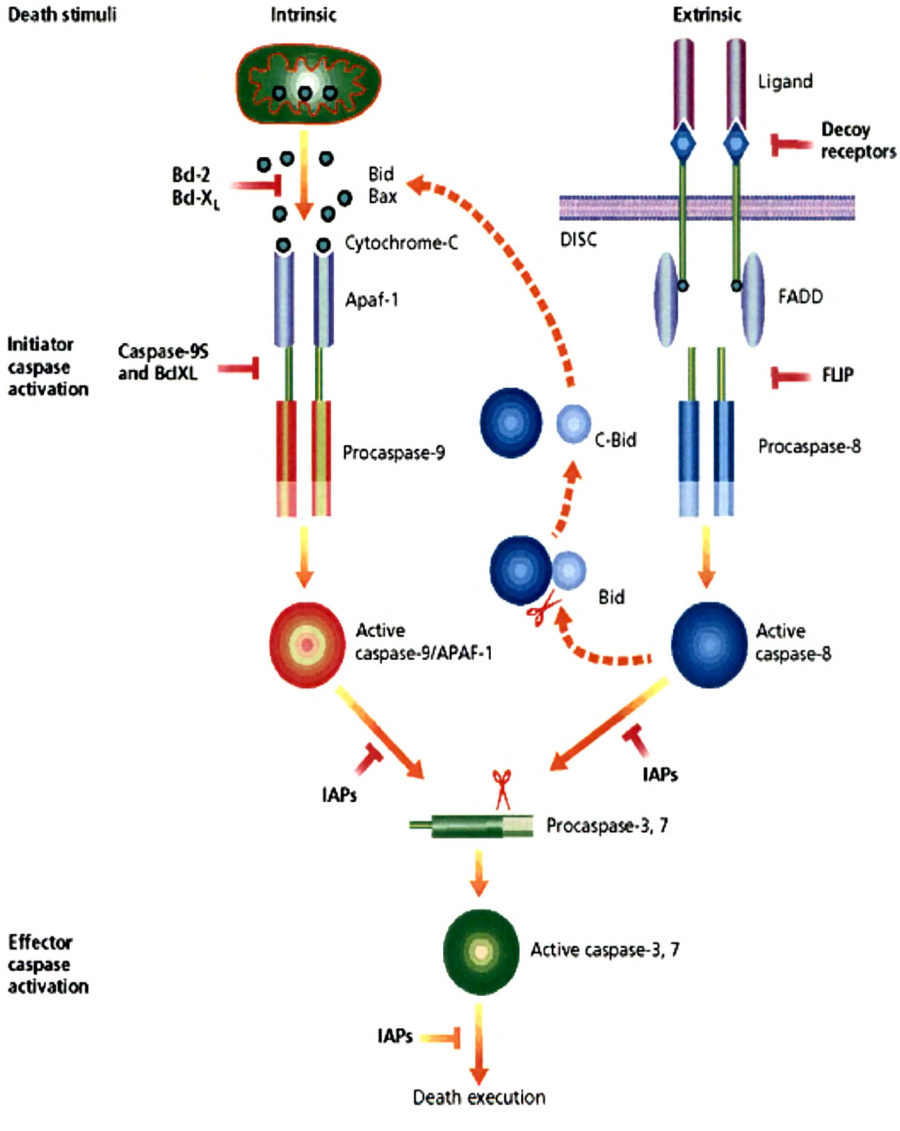


Figure 1.3: Extrinsic and Intrinsic pathways during apoptosis

Mitochondria in apoptosis:

Mitochondria play a key role in apoptotic cell death in mammals, by releasing apoptogenic factors during apoptosis, which are otherwise stored safely inside (Tsujimoto and Shimizu, 2000). It was shown that the mitochondria undergo permeability transition and loss of the mitochondrial transmembrane potential under various apoptotic conditions such as Ca^{2+}overload, and increased oxidant production (hypoxia and reperfusion), leading to the release of two main groups of normally sequestered proapoptotic 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" (Chinnaiyan, 1999; Hill *et al.*, 2004). The clustering of procaspase-9 in this manner leads to caspase-9 activation (Fig. 1.4).

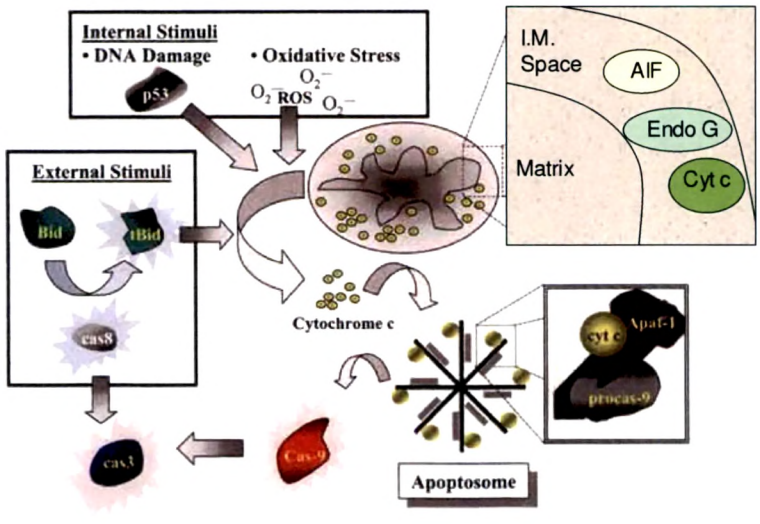


Figure 1.4: Role of Mitochondria in apoptosis

The second group of proapoptotic proteins, AIF and endonuclease G (Endo G) are released from the mitochondria during apoptosis. AIF translocates to the nucleus and causes DNA fragmentation into ~ 50-300 kb pieces and condensation of peripheral nuclear chromatin (Joza *et al.*, 2001). This early form of nuclear condensation is referred

to as "stage I" condensation (Susin *et al.*, 2000; Li *et al.*, 2001). AIF and endonuclease G both function in a caspase independent manner.

The control and regulation of these apoptotic mitochondrial events occurs through members of the Bcl-2 family proteins (Cory and Adams, 2002). The Bcl-2 family proteins govern mitochondrial membrane permeability and can be either proapoptotic or antiapoptotic. Some of the antiapoptotic proteins include Bcl-2, Bcl-x, Bcl-xL, Bcl-xS, Bcl-w, BAG, and some of the proapoptotic proteins include Bax, Bak, Bid, Bad, Bim and Bfk. Balance between these two types of proteins decides whether cell would survive or undergo apoptosis. Bad can heterodimerize with Bcl-xL or Bcl-2, neutralizing their protective effect and promoting cell death (Yang *et al.*, 1995). Fas activated caspase-8 cleaves Bid which then causes alteration in mitochondrial permeability (Li *et al.*, 1998; Esposti, 2002).

Execution Pathway:

The extrinsic and intrinsic pathways merge at the execution phase, the final common pathway of apoptosis. It is the activation of the execution caspases by caspase-9 and caspase-8. Execution caspases activate cytoplasmic endonuclease, which degrades nuclear material, and proteases that degrade the nuclear and cytoskeletal proteins. Cleavage by caspases can either activate or inactivate their substrates; for example, cleavage activates the Rho associated kinase ROCK1, which promotes membrane blebbing (Coleman *et* al., 2001; Sebbagh *et* al., 2001), whereas proteolysis by a caspase inhibits the DNase inhibitor ICAD and unleashes DNA fragmentation by the CAD nuclease (Enari *et al.*, 1998; Liu *et* al., 1997). Caspase-3, caspase-6, and caspase-7 function as effector or executioner caspases, cleaving various substrates including cytokeratins, PARP, the plasma membrane cytoskeletal protein alpha fodrin, the nuclear protein NuMA and others, that ultimately cause the morphological and biochemical changes seen in apoptotic cells (Slee *et al.*, 2001). CAD enters the nucleus and degrades chromosomal DNA (Sakahira *et al.*, 1998). This later and more pronounced chromatin condensation is referred to as "stage II" condensation (Susin *et al.*, 2000). Caspase-3 also induces cytoskeletal reorganization and disintegration of the cell into apoptotic bodies. Phagocytic uptake of apoptotic cells is the last stage of apoptosis. The appearance of phosphatidylserine on the outer leaflet of apoptotic cells (Fig. 1.5) facilitates non

inflammatory phagocytic recognition, allowing for their early uptake and disposal (Fadok *et al.*, 2001).

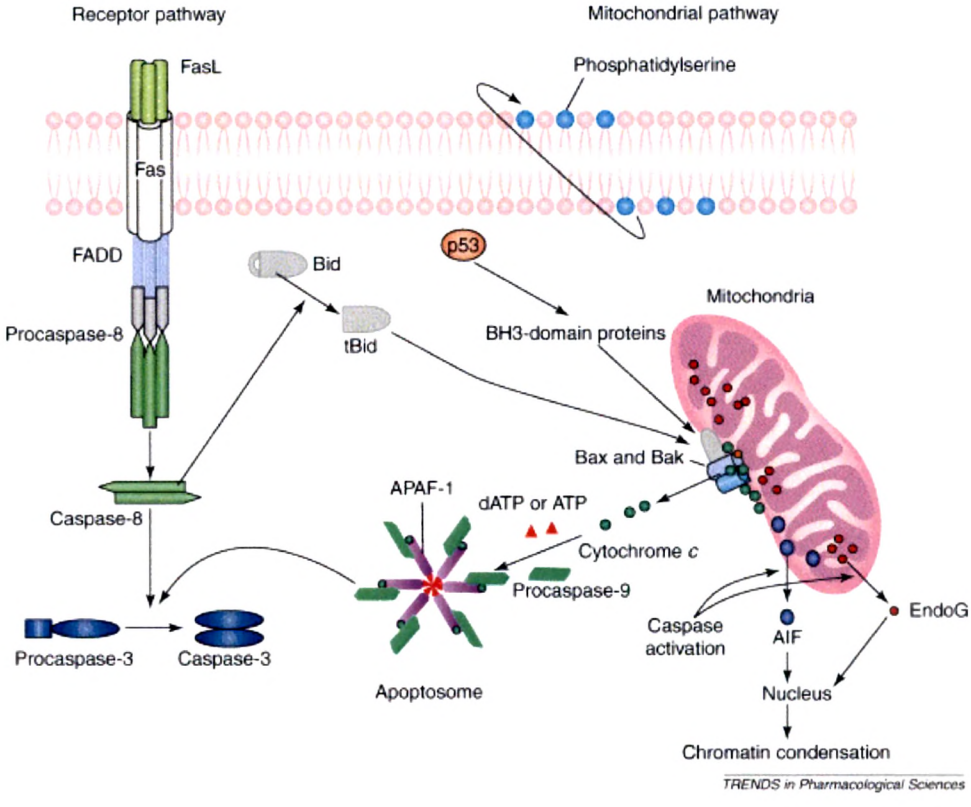


Figure 1.5: Caspase dependent cell death *via* plasma membrane receptor and mitochondrial pathway

1.1.2.2. Type II cell death or Autophagy

Autophagy in Greek means "to eat self". Autophagy, or autophagocytosis, is a catabolic process involving the degradation of a cell's own components through the lysosomal machinery. Autophagy has important roles in developmental processes, human diseases and cellular responses to nutrient deprivation (Schwartz *et al.*, 1993; Gozuacik and Kimchi, 2004; Debnath *et al.*, 2005). It is a major mechanism by which a starving cell reallocates nutrients from unnecessary processes to more essential processes. During starvation, autophagy leads to the breakdown of non vital components and the release of nutrients, ensuring that vital process can continue (Yorimitsu and Klionsky, 2005). Mutant yeast cells that have a reduced autophagic capability rapidly

perish in nutrition deficient conditions (Tsukada and Ohsumi, 1993). Studies with mice have shown that mutation in *atg*7 impairs starvation induced autophagy (Komatsu *et al.*, 2005). In autophagic or type II programmed cell death there is early degradation of organelles but preservation of cytoskeletal elements until late stages. Caspase activation and DNA fragmentation occur very late (if at all) in autophagic cell death. Autophagic cell death is also characterized by lack of chromatin condensation, massive vacuolization of the cytoplasm, and with accumulation of double membrane autophagic vacuoles, with little or no uptake by phagocytic cells.

Types of Autophagy:

Macroautophagy is the sequestration of organelles and long lived proteins in a double membrane vesicle, called an autophagosome or autophagic vacuole (AV), within the cell. The formation of autophagosomes is initiated by class III phosphoinositide 3-kinase (Schmid and Muenz, 2007) and autophagy related gene, *atg* 6 (also known as Beclin-1) (Liang *et al.*, 1999). Expansion of autophagic vesicles is mediated by two ubiquitin like conjugation systems (Mizushima *et al.*, 1998; Ichimura *et al.*, 2000; Ohsumi, 2001):

(1) Atg12 pathway [involving Atg12 (ubiquitin like; known as LC3 in mammalian cells), Atg7 (E1-like), Atg10 (E2-like), and Atg5)]; and

(2) Atg8 pathway [involving Atg8 (ubiquitin like), Atg7 (E1-like), Atg3 (E2-like), and Atg4].

Intra-autophagosomal components are degraded by lysosomal hydrolases. At the same time, LC3-II in autolysosomal lumen is degraded. Thus, lysosomal turnover of the autophagosomal marker LC3-II reflects starvation induced autophagic activity (Tanida *et al.*, 2008).

It seems that the same set of proteins is involved in both autophagic death and autophagy (survival), but their regulation is substantially different during each process. Depletion of amino acids or serum from WT MEFs induces apoptosis, but if apoptosis is blocked (e.g. by Bax/Bak deficiency), activation of autophagy provides the nutrients necessary for cells to survive (Fig. 1.6). Therefore, blocking autophagy causes cell death to be enhanced (Tsujimoto and Shimizu, 2005). Autophagy per se is not sufficient for

autophagic cell death, the outcome is context dependent. It is characterized by the lack of a tissue inflammatory response. Autophagy is part of everyday normal cell growth and development.

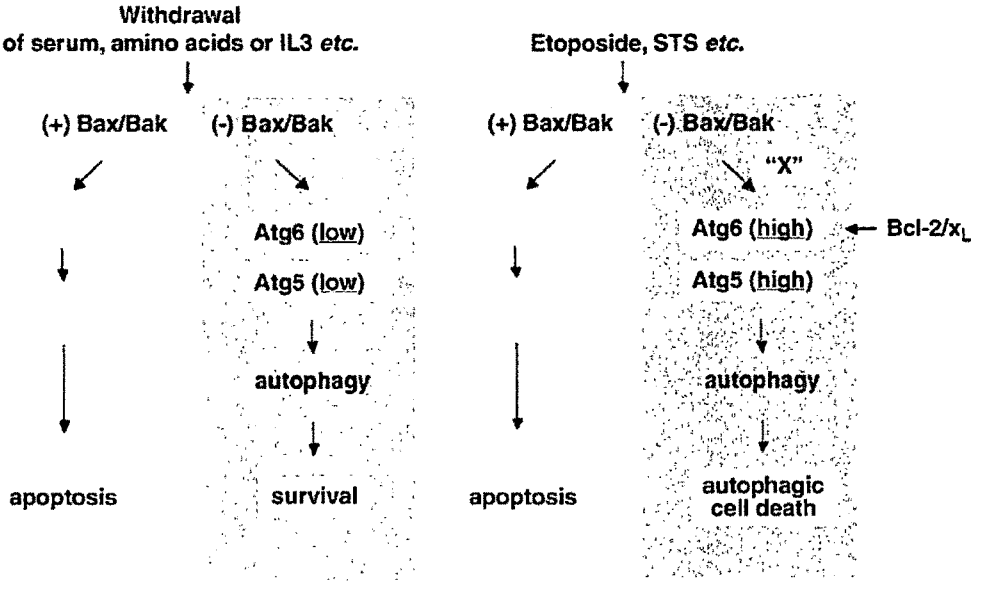


Figure 1.6: Autophagy and autophagic death

Microautophagy, on the other hand, happens when lysosomes directly engulf cytoplasm by invagination, protrusion, and/or septation of the lysosomal limiting membrane. Till date there is no conclusive evidence that autophagy results in the total destruction of cell. Also the cause and consequence relationship between autophagy and cell death has not been established.

1.1.2.3. Type III cell death or Nonapoptotic cell death

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. Most commonly observed caspase independent cell death is paraptosis. Paraptosis appears to occur during the development of the nervous system, as well as in some cases of neurodegenerative disorders (Bredesen *et al.*, 2007).

Paraptosis is a form of programmed cell death that is distinct from apoptosis by the criteria of morphology, biochemical features and response to apoptotic inhibitors. Paraptosis has recently been characterized by cytoplasmic vacuolation that begins with progressive swelling of mitochondria and the endoplasmic reticulum (ER). It typically does not respond to caspase inhibitors nor does it involve activation of caspases, absence of PARP cleavage, the formation of apoptotic bodies, or other characteristics of apoptotic morphology (Sperandio *et al.*, 2000).

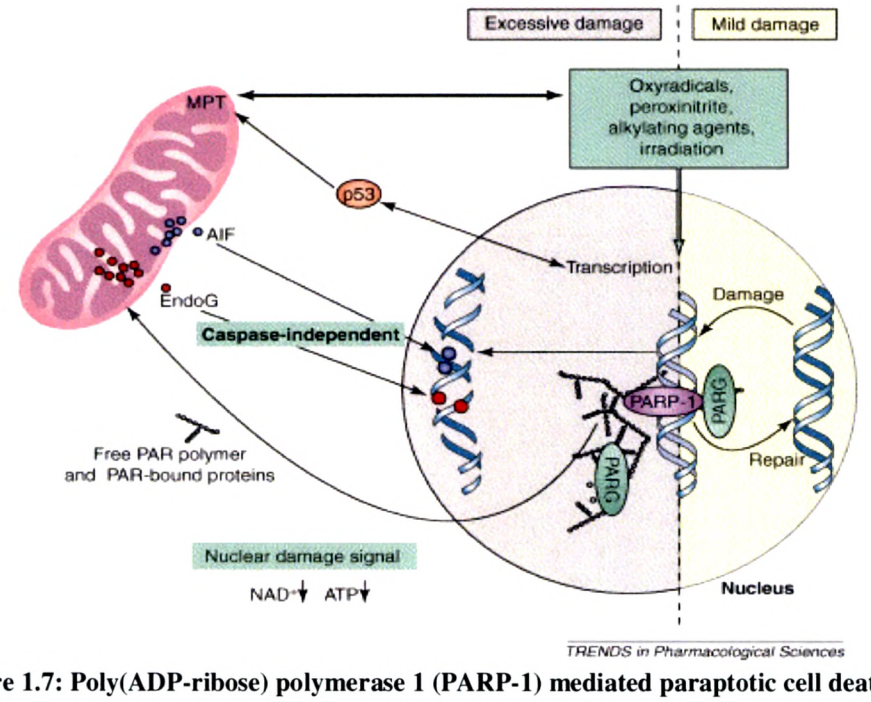


Figure 1.7: Poly(ADP-ribose) polymerase 1 (PARP-1) mediated paraptotic cell death

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. 1.7). 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.

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).

Rat monocytes kill mM-CSF (membrane form of macrophage colony stimulating factor) expressing T9 glioma cells by disrupting ionic homeostasis in the target cell. It demonstrates that osmotic dysregulation of the tumor cells induced by big potassium (BK) channel activation, provides not only the mechanism by which macrophage mediated paraptosis occurs but also how paraptosis underlies subsequent initiation of immune response (Hoa *et al.*, 2009).

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).

Table 1.1: Comparison of apoptosis, necrosis and paraptosis (Sperandio *et al.*, 2000)

	Apoptosis	Necrosis	Paraptosis
Nuclear fragmentation	+	-	-
Chromatin condensation	+	-	+
Vacuolation	-	+	+
Mitochondrial swelling	Sometimes	+	Late
Large scale DNA Fragmentation	+	-	-
PARP cleavage	+	-	-

Organelles involved in Paraptosis:

Mitochondria are central to the life of eukaryotic cells. In addition to their role in cellular energy metabolism, mitochondria are now recognized as central players in cell death as well (Liu *et al.*, 1996). Critical to this role is the mitochondrial permeability transition pore (MPTP) opening of which uncouples mitochondria, preventing them from providing the energy needs of the cell subsequently leading to cell death. Besides apoptosis mitochondria also play a central role in nonapoptotic cell death through the release of proapoptotic proteins contained in the intermembrane space. These include apoptosis inducing factor (AIF) and Smac (or Diablo) in response to an apoptotic stimulus. One possible mechanism by which these proteins are released may be through opening of the MPTP. This causes mitochondrial swelling, rupture of the outer membrane and non specific release of intermembrane proteins and also respiratory inhibition.

OMI/HtrA2 plays a role in caspase dependent cell death, but it can also act as an effector protein in necrosis like PCD. This function is independent of its IAP (inhibitor of apoptosis proteins) binding activity and is mediated via its protease activity (Li *et al.*,

2002). It is, however, difficult to make firm conclusions about the precise contribution of OMI/HtrA2 to cell death, as down-regulation of OMI/HtrA2 expression influences both its mitochondrial function and its cytosolic role in cell death (Saelens *et al.*, 2004).

Another mitochondrial protein that potentially contributes to both caspase independent and caspase dependent cell death is endonuclease G. Endo G is able to induce caspase independent DNA fragmentation in isolated nuclei (Li *et al.*, 2001). It is likely that endonuclease G cooperates with caspase activated exonucleases and DNase I to generate internucleosomal DNA fragments under apoptotic conditions (Widlak *et al.*, 2001; Jaattela, 2004).

Another mechanism for intermembrane protein release involves selective permeation of the outer mitochondrial membrane through recruitment of proteins such as Bax, Bad and Bid. These proteins, either on their own, or through interaction with outer membrane proteins such as the voltage dependent anion channel (VDAC), induce channel formation in the outer membrane. According to the severity of the insult to the cell, this allows mitochondria to determine whether a cell should die, and also the nature of death (Bernardi *et al.*, 1999).

Lysosomes are a component of the degradative machinery in eukaryotic cells. Lysosomes are membrane bound vesicles containing acid hydrolases which include proteases, glycosidases, lipases, and phosphatases (Bechet *et al.*, 2005). Therefore, lysosomes are intracellular compartments dedicated to the degradation of macromolecules. In the classic apoptosis-necrosis paradigm, lysosomes were solely involved in necrotic and autophagic cell death, and the lysosomal proteases were believed to degrade proteins within the lysosome. In recent years, however, it has become evident that the role of lysosomes in cell death is far more sophisticated (Bidere *et al.*, 2003).

The key factor in determining the type of cell death is the magnitude of lysosomal permeabilization and the amount of proteolytic enzymes released into the cytosol (Yuan *et al.*, 2000). Partial, selective permeabilization triggers apoptotic like PCD while massive breakdown of lysosomes results in unregulated necrosis (Bursch, 2001). Lysosome permeability is affected by reactive oxygen species. Experimental evidence suggests that reactive oxygen species–induced lysosomal permeabilization

usually precedes mitochondrial dysfunction, thereby creating a feedback loop in which mitochondrial reactive oxygen species can lead to more lysosomal permeabilization (Zhao *et al.*, 2003).

Cathepsins are a group of aspartate proteases found predominantly in lysosomes. Cathepsin D is the most abundant lysosomal proteases. Cathepsin B and D are the most stable at physiological, cytoplasmic pH and seem to have the most prominent role in apoptotic and necrotic like PCD (Guicciardi *et al.*, 2004; Leist and Jaattela, 2001). An abundant lysosomal cathepsin is the aspartic protease Cathepsin D. Most of the members become activated at the low pH found in lysosomes. Partial destabilization of lysosomal membrane leads to release of Cathepsin D in cytosol. Cathepsin D triggers Bax activation which induces the release of AIF into cytosol (Bidere *et al.*, 2003). It also induces generalized proteolysis leading to caspase independent cell death (Fig. 1.8).

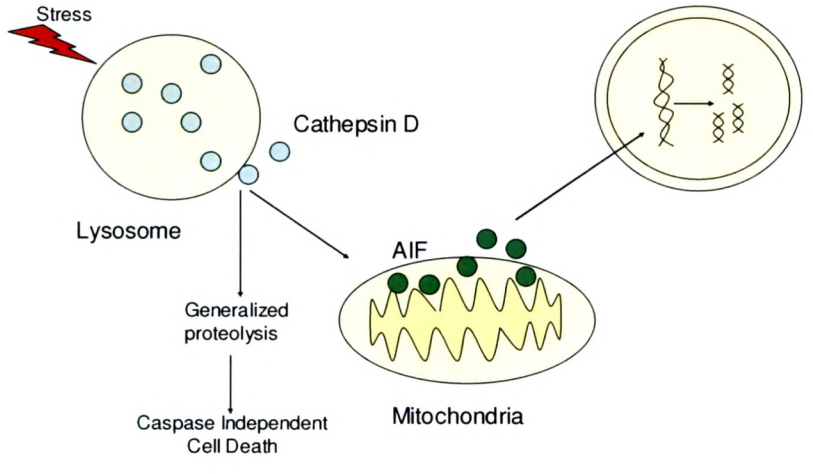


Figure 1.8: Paraptotic cell death induced by Cathepsin D release

Dysregulation of protease expression and their activity in particular is involved in various pathological conditions, such as cardiovascular and neurodegenerative diseases, arthritic, infection and cancer. Therefore, proteases are one of the attractive potential therapeutic targets.

Endoplasmic reticulum is an important sensor of cellular stress that can withhold protein synthesis and metabolism to restore cellular homeostasis (Travers *et al.*, 2000). If the damage to the ER is too extensive, this can initiate PCD via the unfolded

protein response or release of calcium into the cytoplasm (Breckenridge *et al.*, 2003). This leads to activation of caspase 12, possibly via translocation of the Bcl-2 family member Bim to the ER (Morishima *et al.*, 2004). Caspase 12 in its inactive state is localized at the cytosolic face of the ER, but it triggers downstream caspases and apoptosis when it becomes activated (Szegezdi *et al.*, 2003; Rao *et al.*, 2002). Besides caspase12 activation, ER stress can induce permeabilization of the mitochondrial membrane *via* calcium ions and thus activate the classic apoptotic pathway as well as other mitochondrial death pathways (Jimbo *et al.*, 2003). Bcl-2 family proteins as well as cytoplasmic calcium shifts orchestrate the cross talk between the mitochondria and the ER (Annis *et al.*, 2004; Mattson and Chan, 2003). Intracellular calcium influx caused by ER stress induces activation of a family of cytosolic proteases, the calpains (calcium activated neutral proteases), which normally reside in the cytosol as inactive zymogens (Guroff, 1964).

Calpains are a family of calcium dependent, non lysosomal cysteine proteases expressed ubiquitously in mammals and many other organisms. The calpain proteolytic system includes the calpain proteases, the small regulatory subunit (CAPNS1), and the calpain specific inhibitor, calpastatin. Calpains are believed to participate in intracellular signal processing *via* limited proteolysis of their targets. They may play a central role in the execution of apoptosis either upstream or downstream of caspases in glucocorticoid treated and irradiated thymocytes, neuronal cells exposed to various stresses. Calpains have been shown to act downstream of caspase activation and contribute to the degradation phase of camptotecin induced apoptosis in HL-60 cells (Wood and Newcomb, 1999). Sanvicens *et al.*, have shown that both caspases and calpains contribute to oxidative stress induced apoptosis in retinal photoreceptor cells (Sanvicens *et al.*, 2004).

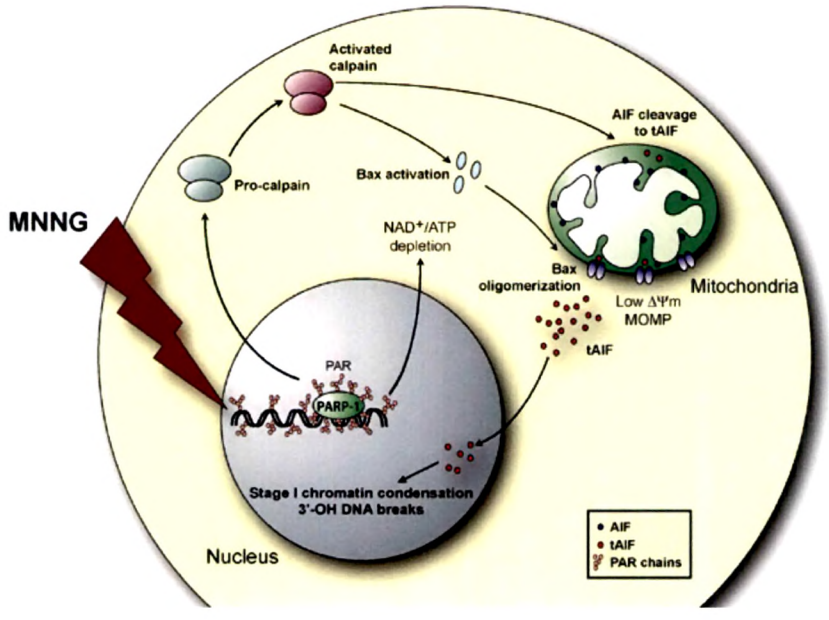


Figure 1.9: MNNG induced DNA damage from PARP-1 to calpain activation

Although the physiological roles of calpains are still poorly understood, they have been shown to be active participants in processes such as cell motility and cell cycle progression, as well as cell type specific functions such as long term potentiation in neurons and cell fusion in myoblasts. Under these physiological conditions, a transient and localized influx of calcium into the cell activates a small local population of calpains (for example, those close to Ca^{2+} channels), which then advance the signal transduction pathway by catalyzing the controlled proteolysis of its target proteins (Fig. 1.9). Calpains have been implicated in apoptotic cell death, and appear to be an essential component of necrosis.

Depending on the type of stimulus or stress cell has an access to different cell death programs. Mitochondria, Lysosomes and ER can be involved in all or certain pathways. The signals from different organelles are linked and may act upstream or downstream of each other (Fig. 1.10).

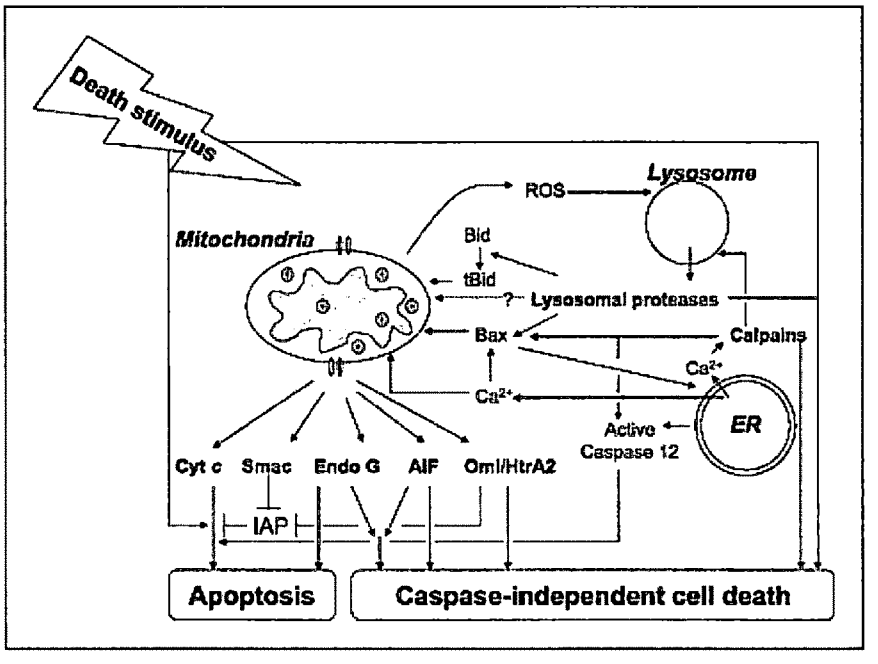


Figure 1.10: Cross talk between cellular organelles during programmed cell death

1.2. Main proteins in Paraptosis

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

1.2.1. POLY(ADP-RIBOSE) POLYMERASE (PARP)

In contrast to well defined mitochondrial mediated cell death, knowledge of nuclear events that participate in cell death processes is limited. Poly(ADP-ribose) polymerase (PARP) plays an important role in activating nuclear mediated paraptosis.

Approximately 40 years ago, Chambon and colleagues reported that the addition of NAD^+ to rat liver nuclear extracts stimulated the synthesis of a polyadenylic acid, which was later identified as poly(ADP-ribose) or PAR. This observation initiated intensive research in the area of poly(ADP-ribosyl)ation and led to the discovery of the first PAR polymerase (PARP) isoform (now termed PARP-1), which was followed by the identification of several other PARP family members (Ame *et al.*, 2004; Virag and Szabo, 2002). In humans there are 17 putative PARP proteins based on protein domain

homology and enzymatic functions (Dawson and Dawson, 2004; Schreiber *et al.*, 2006). There are nine PARPs reported in *D. discoideum* (Otto *et al.*, 2005).

The DNA repair and protein modifying enzyme PARP-1, which is also called poly(ADP-ribose) synthetase and poly(ADP-ribose) transferase, is an abundant nuclear protein that is involved in the DNA base excision repair system (Smith, 2001). PARP is found in all eukaryotes except for yeast in which the expression of human PARP-1 was shown to lead to retarded cell growth (Kaiser *et al.*, 1992). It has been reported that in *S. cerevisiae* genome no DNA sequence similar to known (ADP-ribosyl) transferase/poly(ADP-ribose) polymerase genes has been found (Rouleau *et al.*, 2004).

Many PARP homologues have been described in humans i.e. PARP-1, PARP-2 (Ame *et al.*, 1999), Vault PARP (Kickhoefer *et al.*, 1996) and Tankyrase-1 (Smith *et al.*, 1998). Each member of the PARP family shares homology on the C-terminal catalytic domain of PARP-1.

PARP-1 Structure:

PARP-1 is a 116 kDa protein. It contains three functionally distinct domains: an amino terminal DNA binding domain (DBD), an auto modification domain (AD) and a carboxyl terminal PARP homology domain that includes the catalytic domain (CAT) (Fig. 1.11) responsible for PAR formation (Mazen *et al.*, 1989; de Murcia *et al.*, 1994; Ruf *et al.*, 1996; Smith, 2001). AD contains acceptor amino acids for the covalent attachment of PAR (Schreiber *et al.*, 2006). Recent studies identified a weak leucine-zipper motif in the amino-terminal region of the AD, which suggests that this motif might be involved in homo and/or hetero-dimerization (D'Amours *et al.*, 1999). DBD contains two structurally and functionally unique zinc fingers (FI: aa, amino acid, 11–89; FII: aa 115–199) (D'Amours *et al.*, 1999; Hassa *et al.*, 2006). Recently, a third and so far unrecognized zinc binding motif is discovered (FIII: aa 233–373) (Langelier *et al.*, 2008; Tao *et al.*, 2008). The DBD also contains a bipartite nuclear localization signal (NLS) that targets PARP-1 to the nucleus (Schreiber *et al.*, 1992). The CAT domain has been suggested to catalyze at least three different enzymatic reactions: the attachment of the first ADP-ribose moiety onto an acceptor amino acid (initiation reaction), the addition of further ADP-ribose units onto already existing ones (elongation reaction) and the generation of branching points (branching reaction) (Ruf *et al.*, 1996). The active site is

formed by a phylogenetically well-conserved sequence of ~50 residues (aa 859–908 of hPARP1). This 'PARP signature' contains the NAD^+ acceptor sites and critical residues involved in the initiation, elongation and branching of PAR.

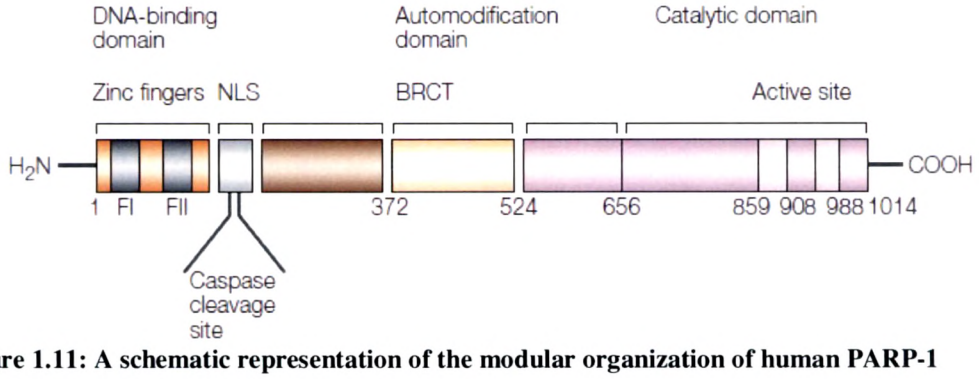


Figure 1.11: A schematic representation of the modular organization of human PARP-1

PARP activity:

Approximately one molecule of PARP-1 is present per 1000 bp of DNA. PARP-1 functions as a DNA damage sensor. It binds with high affinity and in a cooperative manner to various DNA structures (for example, cruciform, curved or supercoiled structures), which influence basal PARP activity (Sastry and Kun, 1990). A basal low level DNA strand breakage due to ROS might have a role in maintaining basal PARP-1 activity.

In response to DNA damage, PARP-1 activity is rapidly increased up to 500-fold upon binding to DNA strand nicks and breaks. On binding to damaged DNA, PARP-1 forms homodimers and catalyzes the cleavage of NAD^+ into nicotinamide and ADP-ribose and then uses the latter to synthesize branched polymers of poly (ADP-ribose) (PAR) covalently attached to proteins (de Murcia *et al.*, 1994; Smith, 2001) (Fig. 1.12). Elongation of the polymeric chain occurs at the 2'-OH of the mono (ADP-ribose). PARP-1 transfers 50–200 residues of PAR to itself and to acceptor proteins such as histones, DNA polymerases, topoisomerases, DNA ligase-II, high-mobility-group proteins and transcription factors (Smulson *et al.*, 2000; Shall and de Murcia, 2000). Poly(ADP-ribosyl)ation of acceptor proteins by PARP-1 modifies the charge distribution of acceptor proteins and increases the hindrance of proteins by the addition of a bulky and complex structure. This modification might inhibit the physiological function of

acceptor proteins and, thus poly(ADP-ribosyl)ation of key cellular proteins might lead to cell death (D'Amours *et al.*, 1999). The synthesis and turnover of ADP-ribose polymers is a dynamic cellular response to DNA damage.

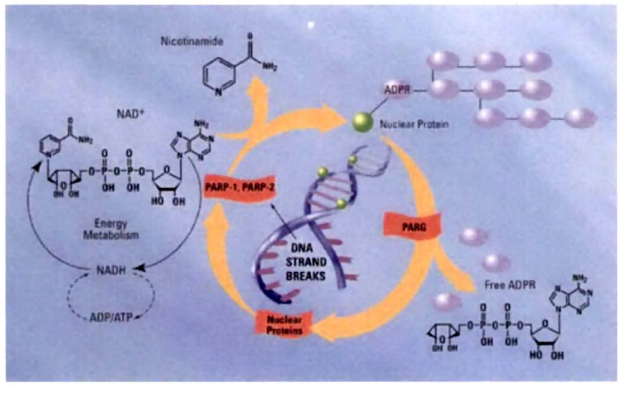


Figure 1.12: PARP upon activation utilizes NAD^+ to synthesize PAR residues

PARylation sites:

Glutamate and lysine in the target proteins and auto modification domain of PARP are the acceptor sites for PAR residues (Fig. 1.13). Recent report suggests that only lysine is the acceptor site for ADP-ribosylation (Altmeyer, 2009). *In vivo* the most abundantly poly(ADP-ribosylated) protein is PARP-1 itself, this represents a major regulatory mechanism resulting in the downregulation of the enzyme activity.

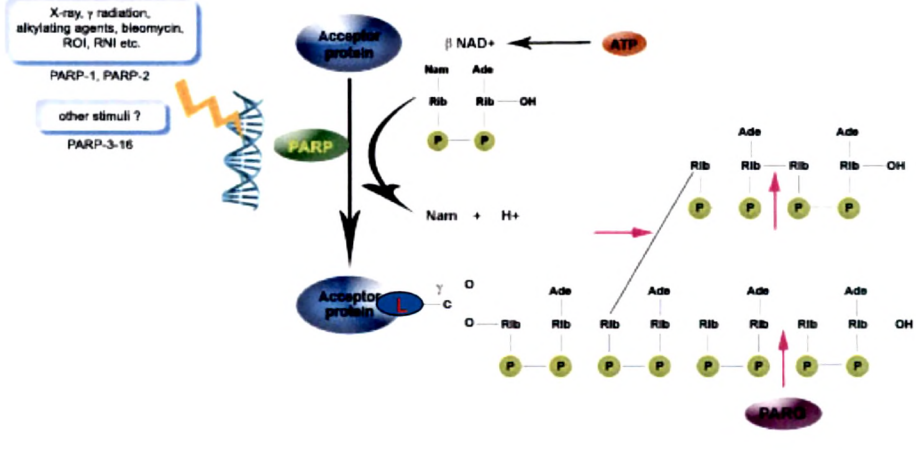


Figure 1.13: Target proteins are PARylated at Lysine (L) residues

Histones are also found to be major acceptors for the PAR residues (Tanuma *et al.*, 1985; Nagele, 1995). Addition of PAR residues confers negative charge to histones, leading to electrostatic repulsion between DNA and histones. This process has been implicated in chromatin remodeling, DNA repair and transcriptional regulation.

Table 1.2 - Examples of target proteins for PARP

Potential function	Acceptors
Modulation of chromatin structure, repair	Histone H1, H2A, H2B, H3 and H4, Topoisomerase I and II, HMG proteins, PARP.
DNA synthesis, repair	DNA ligase I and II (*in vitro*), DNA polymerase α,β (*in vitro*), Terminal transferase, PCNA.
Transcription	RNA polymerase I and II, hnRNP, Fos, p53, NF-KB

(Table adapted from D' Amours *et al.*, 1999)

Functions of PARP:

The biological role of PARP is complex and involves the following main functions:

DNA repair and maintenance of genomic integrity

PARP-1 has been implicated in DNA repair and maintenance of genomic integrity (de Murcia and de Murcia, 1994; de Murcia *et al.*, 1997; Schreiber *et al.*, 1995; Chatterjee *et al.*, 1999; Shall and de Murcia, 2000). This "guardian angel" function is indicated by delayed DNA base excision repair and by a high frequency of sister chromatid exchange in PARP-1 deficient cells exposed to ionizing radiation or treated with alkylating agents (de Murcia *et al.*, 1997). The nuclear accumulation of PAR leads to the recruitment of DNA repair proteins (Fig. 1.14) such as XRCC1, DNA ligase III, DNA polymerase ε and the Ku70 subunit of the DNA dependent protein kinase (Pleschke *et al.*, 2000; Malanga and Althaus, 2005). More recently, the DNA damage

responsive kinase, ATM was added to the list of PAR binding proteins (Haince *et al.*, 2007).

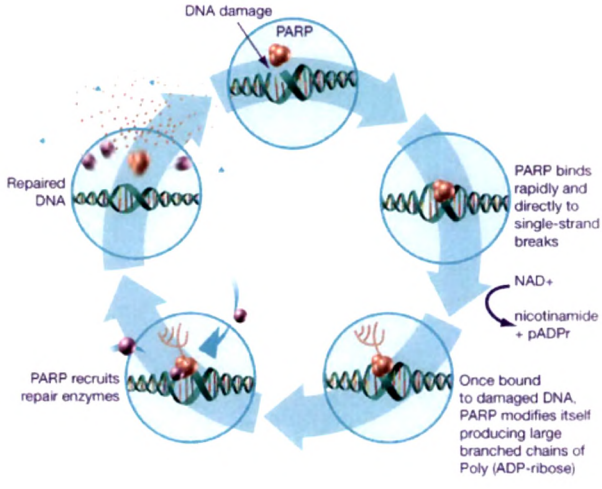


Figure 1.14: PARP detects the presence of nicks or damage and PARylate the target proteins

Regulation of replication and differentiation

The involvement of PARP-1 in the regulation of replication is supported by observations that poly(ADPribose) metabolism is accelerated in the nuclei of proliferating cells (Tanuma *et al.*, 1978; Kanai *et al.*, 1981; Leduc *et al.*, 1988; Bakondi *et al.*, 2002). Furthermore, PARP-1 is part of the multiprotein replication complex (MRC) that catalyzes viral DNA replication *in vitro* by PARylating few MRC proteins (Simbulan-Rosenthal *et al.*, 1999). Poly(ADP-ribosylation) has been implicated in the regulation of telomerase activity (Smith and de Lange, 2000).

Gene expression

PARP-1 also regulates the expression of various proteins at the transcriptional level. Three principal mechanisms are involved: first, the effects of PARP on histones, which are followed by changes in the chromatin structure; second, the regulation of DNA methylation by PARP; and third, the participation of PARP in enhancer/promoter binding complexes. PARP-1 can regulate the activity of promoters directly (Soldatenkov

et al., 2002). PARP induces dissociation of nucleosomes and decondensation of chromatin, which facilitates the transcription of target genes (Chiarugi, 2002). PARP adds ADP-ribose units to transcription factors, which prevent them rebinding to DNA (Tulin and Spradling, 2003). Of special importance is the regulation by PARP-1 of the production of inflammatory mediators such as the inducible nitric oxide synthase (iNOS) (Hauschildt *et al.*, 1992; Le Page *et al.*, 1998; Szabo *et al.*, 1998; Oliver *et al.*, 1999). PARP-1 also forms a complex with nuclear factor kB (NF-kB) and can activate the expression of NF-kB target genes (Ullrich *et al.*, 2003). Recent studies in *Drosophila* reveal that during transient activation of gene expression in response to an environmental stimulus (heat shock), PARP is recruited to condensed chromatin (Tulin and Spradling, 2003).

PARP-1 is also required for the activation of other inflammation related transcription factors, such as activator protein-1 (AP1), AP2, transcription enhancer factor-1 (TEF1), trans-acting transcription factor-1 (SP1), octamer-binding transcription factor-1 (Oct1), yin-yang-1 (YY1) and signal transducer and activator of transcription-1 (STAT1) (Kraus and Lis, 2003; Virag and Szabo, 2002). Thus PARP might be recruited to promoters or contact enhancers directly by recognizing certain DNA structures or sequences (Jagtap and Szabo, 2005).

Cell cycle regulation

PARP deficient cells have disturbed cell cycle progression and establish a tetraploid population. PARP-1 regulates E2F-1 expression during S-phase re-entry when quiescent cells re-enter the cell cycle (Simbulan-Rosenthal *et al.*, 2001; 2003).

Cytoskeletal organization

Poly(ADP-ribosylation) may also be involved in the regulation of cytoskeletal organization. A recent study reported morphological alterations in *Drosophila* overexpressing PARP-1 (Uchida *et al.*, 2001). The overexpression of PARP-1 disrupted the organization of cytoskeletal F-actin, resulting in aberrant cell and tissue morphology.

Energy source

PAR polymer has been identified as an emergency source of energy used by the base excision machinery to synthesize ATP. The concerted action of PARG and ADP-ribose pyrophosphorylase is capable of generating ATP from ADP-ribose units (Maruta *et al.*, 1997). Ziegler's group showed that Poly(ADP-ribose), was converted to ATP by pyrophosphorylytic cleavage utilizing the pyrophosphate generated from dNTPs during DNA synthesis. The adenylyl moiety was then preferentially used to adenylate DNA ligase III, then it was transferred to the 5′-phosphoryl end of the nicked DNA. Finally, ligation to the 3′-OH end resulted in the release of AMP (Oei and Ziegler, 2000).

Protein degradation

Poly(ADP-ribose) may also serve as a signal for protein degradation in cells subjected to oxidative stress (Ciftci *et al.*, 2001; Ullrich and Grune, 2001). 20S proteasome activity was upregulated in H_2O_2 treated cells and proteasome becomes PARylated. This resulted in degradation of PARylated histones (Ullrich and Grune, 2001).

In addition to PARP catalyzed covalent poly(ADP-ribosylation), poly(ADP-ribose) polymers can non covalently bind to specific $(ADP\text{-}ribose)_n$ binding motifs in proteins, such as histones, XRCC1, p53, and DNA polymerase ε, and thereby modify their function (Althaus *et al.*, 1993; Pleschke *et al.*, 2000). PARP-1 mediated poly(ADP-ribosyl)ation also regulates malignant transformation, cellular division and mitochondrial function (Chiarugi, 2002).

PARP and cell death

PARP has been implicated in both apoptosis and necrosis. PARP is recognized as the 'death substrate', because PARP-1 was one of the first identified substrates of caspases. During apoptosis, caspase 7 and caspase 3 cleave PARP1 into two fragments: p89 and p24. The cleavage of PARP-1 separates its DBD from its catalytic domain, which inactivates the enzyme. Thus cleavage of PARP is one of the hallmark features of apoptosis. The normal development pattern that is seen in PARP-1 deficient mice is indicative that PARP-1 does not have an active role in apoptosis. In fact, PARP cleavage prevents the overactivation of PARP (and the PARP-dependent cellular energetic crisis)

and thereby maintains cellular energy for certain ATP dependent steps of apoptosis (Herceg and Wang, 1999; Aikin *et al.*, 2004). Excessive DNA damage causes PARP overactivation leading to depletion of cellular NAD^+/ATP pools biasing the cell towards necrosis. Thus extent of DNA damage regulates PARP activity which in turn decides the fate of a cell (Fig. 1.15).

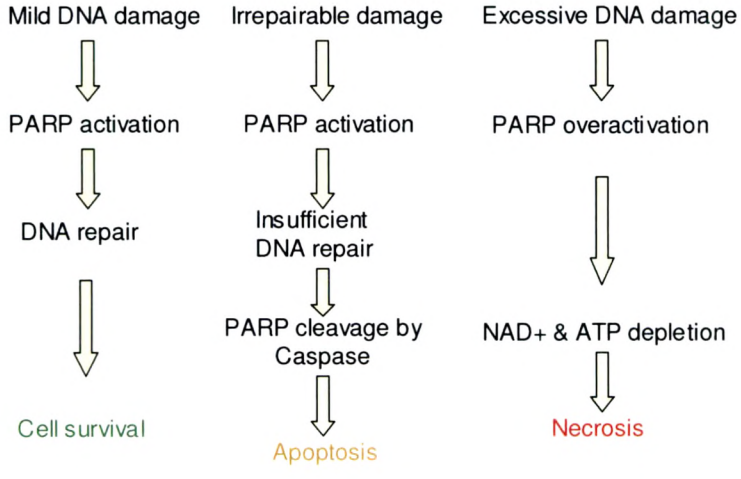
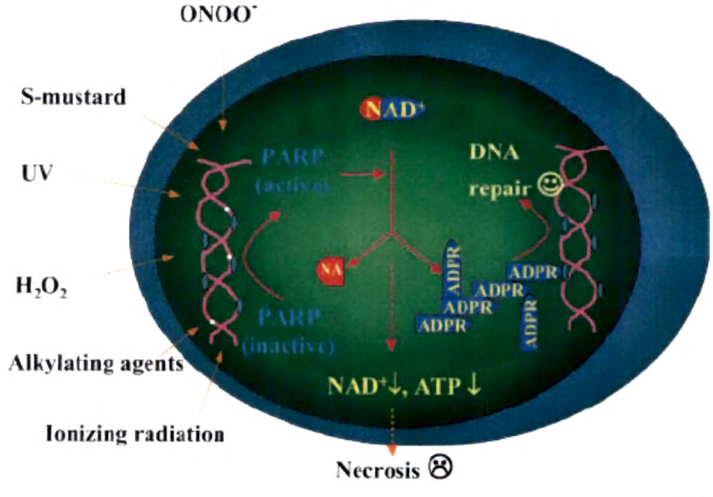


Figure 1.15: PARP determines the fate of a cell

PARP mediated cell death is caused by oxidative insult (H_2O_2, peroxynitrite) (Fig. 1.16), excitotoxic injury (NMDA, glutamate), complex I inhibition and ischemia/reperfusion (Virag and Szab, 2002; Woon and Threadgill, 2005). Nitric oxide, through the generation of the cytotoxic oxidant peroxynitrite (which is formed when nitric oxide reacts with superoxide), is one of the principal pathophysiological triggers of DNA injury and PARP activation (Szabo *et al.*, 1996; Zingarelli *et al.*, 1996).

Oxidant induced DNA damage also accumulates mono ADP-ribose residues which serve as second messenger to induce gating of the transient receptor potential channel, melastatin subfamily (TRPM) Ca^{+2} channels (Buelow *et al.*, 2008; Yang *et al.*, 2006). PARP inhibitors and PARP1 gene disruption can reduce cell death resulting from oxidative stress (Schraufstatter *et al.*, 1986), radiation (Piela-Smith *et al.*, 1992), nitric oxide, peroxynitrite (Zhang *et al.*, 1994; Szabo and Dawson, 1998), and other agents that damage DNA (Ha and Snyder, 1999). Oxidative stress contributes to cell death in cerebral ischemia (Dawson and Dawson, 1998) and genetic or pharmacological inhibition of PARP-1 reduces ischemic cell death (Eliasson *et al.*, 1997). PARP-1

inactivation can also prevent neuronal death induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Mandir *et al.*, 1999) and other oxidants (Szabo and Dawson, 1998).



(Virag *et al.*, 1999)

Figure 1.16: PARP activation in response to DNA damaging agents and cell death

PARP activation induced depletion of the cellular pyridine nucleotide pool (Fig. 1.16) impairs important NAD^+ dependent cellular pathways, including glycolysis and mitochondrial respiration. NAD^+ deficiency allows only the ATP consuming part of anaerobic glycolysis to take place, thereby decreasing the synthesis of pyruvate and the mitochondrial formation of NADH. NADH deficient mitochondria undergo depolarization, which converts the ATP synthase into an ATPase. Extensive activation of PARP-1 leads to glycolytic blockade, energy failure, and cell death. Repletion of intracellular NAD^+ completely restored glycolytic capacity and prevented cell death. These results suggest that NAD^+ depletion is the cause of glycolytic failure after PARP-1 activation (Ying *et al.*, 2003).

PARP mediated cytotoxicity involves mitochondrial changes and further downstream events. A recent report suggests that PARP-1 localizes to mitochondria and, thus, could directly mediate these effects (Du *et al.*, 2003). However, other investigations using carefully controlled sub cellular fractions have not observed mitochondrial localization of PARP-1 (Yu *et al.*, 2002).

Energy depletion may not be a primary factor in PARP-1 mediated cell death (Fossati *et al.*, 2007; Moubarak *et al.*, 2007). Long chain PAR itself is a key player in caspase independent cell death. PARP-1 induced cell death involves PAR polymer formation and requires AIF nuclear translocation (Andrabi *et al.*, 2006; Yu *et al.*, 2002, 2006). PARP dependent cell death was accompanied by a reduction of mitochondrial transmembrane potential, nuclear accumulation of apoptosis inducing factor (AIF) and large scale DNA fragmentation.

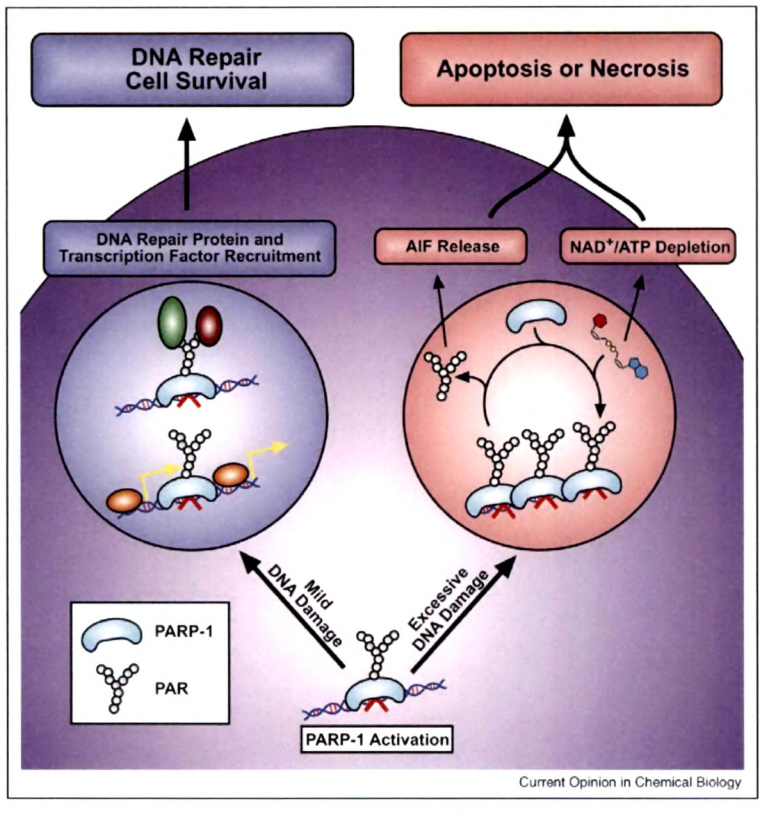


Figure 1.17: The biological functions of PARP-1 signaling in response to DNA damage

Evidence showed that PAR polymer injected into living cells induces AIF release from mitochondria and translocation to nucleus (Yu *et al.*, 2006). Neutralizing antibodies to PAR or catabolism of PAR by overexpression of PARG prevented AIF translocation to the nucleus and cell death (Andrabi *et al.*, 2006; Yu *et al.*, 2006). These results reveal that PAR polymer is an AIF releasing factor (Fig. 1.17), which plays important roles in PARP-1 dependent cell death. Recently, it has been showed that AIF is a PAR polymer binding protein determined by Liquid chromatography mass

spectrometry (LC-MS/MS) analysis (Gagne *et al.*, 2008). The connection between PARP-1 and AIF was first described in 2002, wherein embryonic fibroblasts from PARP-1 knockout mice failed to release AIF from mitochondria and cytoprotection was conferred against DNA damaging agents (Yu *et al.*, 2002). This PARP/AIF axis mediates caspase independent cell death.

Regulation of PARP activity

As mentioned earlier poly(ADP-ribosylation) is a dynamic process, following PARP activation the appearance of poly(ADP-ribose) is transient because it will rapidly be degraded by poly(ADP-ribose) glycohydrolase (PARG) (Heeres and Hergenrother, 2007). Two enzymes, poly(ADP-ribose) glycohydrolase (PARG) and ADP-ribosyl lyase are involved in the catabolism of PAR. The linear and branched portions are cleaved by PARG and lyase removes the protein proximal ADP-ribose monomers (Davidovic *et al.*, 2001). Previously, poly(ADP-ribose) was initially believed to be produced in response to DNA damage, recent data suggest that the synthesis and degradation of poly(ADP-ribose) is a death signal in variety of diseases which include DNA damage (Beneke *et al.*, 2004; Heeres and Hergenrother, 2007). The PAR polymer is thus a central player in both life and death of the cell. PAR is required for proper mitosis, and PAR synthesis helps to stimulate the DNA damage repair response. Conversely, too much PAR synthesis may deplete NAD^+/ATP stores and kill the cell, and long chain PAR itself is a key player in caspase independent cell death.

The regulation of PARP-1 activity is established through different mechanisms. The best characterized mechanism is the regulation of enzyme activity through auto-poly-ADP-ribosylation (Kawaichi *et al.*, 1981). Furthermore, nicotinamide, the smaller cleavage product of NAD^+, also exerts inhibitory effect on PARP-1, allowing negative feedback regulation. Phosphorylation of PARP by protein kinase C also results in enzyme inhibition (Tanaka *et al.*, 1987; Bauer *et al.*, 1992).

As already mentioned that PARP utilizes NAD^+ as its substrate and forms PAR polymer, this has led to the use of PARP inhibitors in the form of nicotinamide analogs such as benzamide to inhibit PARP activity. Benzamide competes with NAD^+ for the catalytic domain of PARP. This activity of benzamide has been exploited in treatment of leukemic cells having telomerase activity depending on PARP (Ghosh *et al.*, 1995).

1.2.2. Poly (ADP-ribose) glycohydrolase (PARG)

Poly(ADP-ribose) glycohydrolase (PARG) is the major catabolic enzyme responsible for the catabolism of poly(ADP-ribose), a reversible covalent-modifier of chromosomal proteins. In contrast to PARP, little is known about PARG in cell function. It was discovered by Miwa and Sugimura in 1971. The mechanism by which PARP activation leads to cell death appears to link with rapid utilization of NAD^+ during formation of poly ADP-ribose (PAR) residues. PAR has a fast turn over rate, with a half life approaching 1 minute due to rapid degradation by the poly (ADP-ribose) glycohydrolases (PARG). It rapidly hydrolyzes the ribose-ribose bonds of both linear and branched portions of PAR bound to acceptor proteins (Davidovic *et al.*, 2001).There are however, atleast two mechanisms by which PARG could influence PARP-mediated cell death and suggests that PARP mediated cell death requires the concomitant action of PARG. Firstly, PARG inhibition could slow the turnover of PAR. Secondly, PARG inhibition could prevent the removal of PAR and hence the inhibition of PARP. This mechanism thereby leads to NAD depletion.

PARG Structure:

PARG is a 110 kDa protein that is ubiquitously expressed in mammalian cells. A 59 kDa cleavage product also has catalytic activity. Human PARG consists of 18 exons and 17 introns. It has a regulatory N-terminal domain and a catalytic domain (Fig. 1.18) (Bonicalzi *et al.*, 2005). The full length 110 kDa PARG is mainly localized to the nucleus. There are no reports on *Dictyostelium* PARG except for dictybase genome.

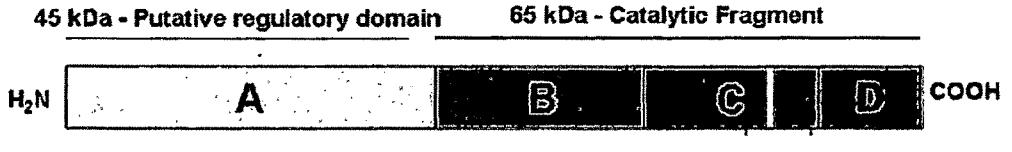


Figure 1.18: Schematic representation of PARG

PARG Function:

PARG functions as an endo and exoglycosidase to rapidly degrade poy(ADP-ribose) and has no other known cellular function. PAR catabolism in the nucleus could

be regulated, at least in part, by a nuclear-cytosolic shuttling of PARG. PARG shuttling to and from the nucleus is probably a very efficient and rapid process that would make it very difficult for the actual detection of PARG translocation. Supporting this notion, Winstall *et al.,* (1999) demonstrated that nuclear PAR (induced after DNA damage) was undetectable in cells overexpressing PARG despite the fact that overexpressed PARG was observed exclusively in the cytosol. Sequence analysis of PARG proteins reveals a potential nuclear export signal (NES) sequence within the N-terminal portion of PARG at amino acid positions 124–132 that is conserved among mammals as well as a functional nuclear localization signal (NLS) (Lin *et al.,* 1997; Shimokawa *et al.,* 1999). Thus, a balance between the NLS and NES signals on PARG probably controls its nuclear and cytosolic localization. Since poly(ADP-ribosyl)ation of nuclear proteins is an ongoing process within cells, PARG probably constantly shuttles between the nucleus and cytosol depending upon PARP-1 activity.

PAR Metabolism:

PARG is capable of hydrolyzing both terminal ADP-ribose units from poly(ADP-ribose) polymers due to its exoglycosidic activity and removes larger oligo(ADP-ribose) fragments *via* endoglycosidic cleavage (Fig. 1.19) (Brochu *et al.,* 1994; Davidovic *et al.,* 2001). Because the Km value of PARG is much lower for larger $(ADP\text{-}ribose)_n$ polymers than for smaller ones, the enzyme probably removes and catabolizes bigger fragments first. PARG then switches to exoglycosidic mode and removes ADP-ribose units one by one. The proximal ADP-ribose moiety is removed from the acceptor proteins by ADP-ribosyl protein lyase (Oka *et al.,* 1984). The high specific activity of PARG compensates for the low abundance of the enzyme (Hatekayama *et al.,* 1986).

Disruption of the *parg* gene by targeting exon 4 in the germline of mice, leading to complete suppression of functional PARG, causes early embryonic lethality (Koh *et al.,* 2004) due to PAR accumulation. In fact, trophoblast stem cell lines derived from these *parg*-null early embryos are viable, but only in the presence of PARP inhibitors (Koh *et al.,* 2004). This suggests that PARP activity is essential for survival. PARG-deficient adult flies show neurodegeneration, reduced locomotor activity and a short

lifespan, suggesting the involvement of PARG in normal neuronal cell metabolism and aging (Hanai *et al.*, 2004).

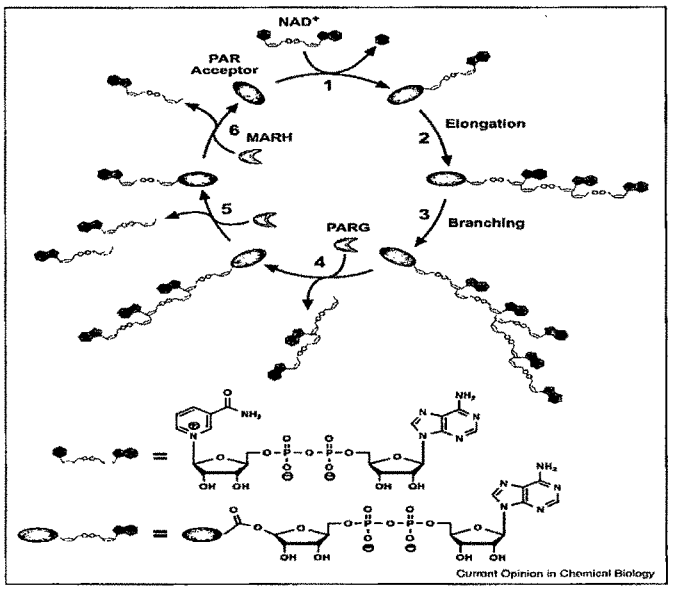


Figure 1.19: The biosynthesis and degradation of PAR

Depending on the therapeutic goal, enhancement or reduction in cell death could be accomplished by modulating PAR levels and one way to do this would be the inhibition of PARG, as it is the key enzyme involved in the degradation of PAR. Keeping with this life/death duality of PARP, the evidence presented below indicates that under certain conditions PARG inhibition could be cytoprotective and under other conditions it could be cytotoxic.

It has been reported that the longest PAR polymers are the most toxic, and there is at least a 40 fold difference in Km values for PARG against long branched polymers versus short chain polymers (Hatakeyama, 1986). It may be possible to primarily inhibit the slow reaction, thus allowing for the PARG mediated degradation of toxic long chain PAR, but still preventing NAD^+/ATP depletion. Still extensive work is required to identify potent molecules to specifically inhibit PARG. Scientists have developed significant interest in the development of potent and cell permeable inhibitors of PARP and PARG.

1.2.3. Apoptosis Inducing Factor (AIF)

AIF protein is highly conserved among mammalian species and it has three domains: an amino-terminal mitochondrial localization sequence (MLS) of 100 amino acids, a spacer sequence of 27 amino acids, and a carboxy terminal 485 amino acid domain with strong homology to oxidoreductases from other vertebrates (*Xenopus laevis*), nonvertebrate animals (*Caenorhabditis elegans*, *Drosophila melanogaster*), plants, fungi, eubacteria, and archaebacteria. The mature AIF protein is a flavoprotein with significant homology to plant ascorbate reductases and bacterial NADH oxidases. AIF is normally confined to the mitochondrial intermembrane space.

The cell death pathway initiated by PARP-1 activation appears to be mediated by AIF. PARP-1 activators, including the DNA alkylating agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and N-methyl-D-aspartate (NMDA) induce the translocation of AIF from mitochondria to the nucleus, nuclear condensation, phosphatidylserine exposure, dissipation of the mitochondrial membrane potential and cell death. Each of these events is caspase independent because broad spectrum caspase inhibitors do not block these events (Yu *et al.*, 2002). However, each process is prevented by the presence of PARP-1 inhibitors and in PARP-1 knockouts. Translocation of AIF occurs rapidly after PARP-1 activation and precedes cytochrome c release and caspase activation. Although caspases might be involved in facilitating cell death, they are not required because broad spectrum caspase inhibitors do not prevent PARP-1 mediated cell death. By contrast, neutralizing antibodies to AIF do block PARP-1 dependent cell death (Yu *et al.*, 2006). Taken together, these results indicate that AIF is an essential downstream effector of the cell death program initiated by PARP-1. AIF translocates from mitochondria to the nucleus where it mediates chromatin condensation and large scale DNA fragmentation, possibly by binding to DNA. In addition to caspase activation, mitochondrial permeability transition, disruption/dissipation of mitochondrial membrane potential, activation and oligomerization of Bax and Bak might induce the release of AIF. Therefore AIF release from mitochondria can be independent of caspases. There are no known inhibitors of AIF. The broad spectrum cytoprotective molecules such as Bcl-2, and a member of the heat shock protein 70 family can delay or prevent AIF mediated toxicity, but their mechanism of action is unknown. Pharmacological agents that hinder

the translocation of AIF or inhibit the promotion of DNA fragmentation induced by AIF might have tremendous therapeutic potential.

AIF also has other mitochondrial function and its structural similarity to flavoproteins indicates that AIF acts as an oxidoreductase in mitochondrial electron transport (Lipton *et al.*, 2002). Cerebellar granule cells from Harlequin (Hq) mice in which AIF expression is reduced markedly are susceptible to oxidative stress. The mitochondrial localization, sensitivity to oxidative stress in Hq mice and NADH oxidase activity of AIF indicates that AIF might be involved in scavenging reactive oxygen species in normal cells. Mutation of the oxidoreductase domain does not alter the death promoting activity of AIF activity following translocation to the nucleus, indicating that this function of AIF is not directly involved in cell death. Although AIF might function as a free radical scavenger to prevent apoptosis under normal physiological circumstances, it is also evident that AIF is an important factor for apoptosis. Cortical neurons from Hq and wild type mice are equally sensitive to hydrogen peroxide induced injury. Although the translocation of AIF was not determined, there is sufficient AIF in Hq neurons to mediate AIF dependent cell death (Joza *et al.*, 2002).

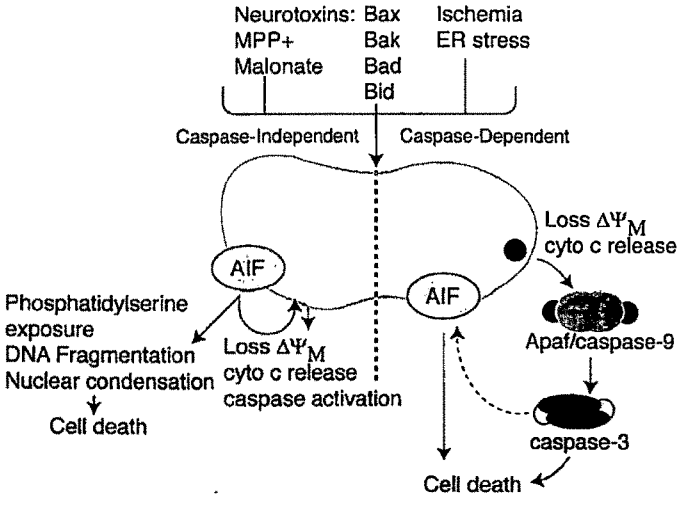


Figure 1.20: Role of AIF in caspase dependent and independent cell death

Parallels can be drawn between AIF and cytochrome c as both are important for cell viability when they are located in mitochondria, but when either is released from the mitochondria, they activate death programs. The activation of mitochondrial membrane transition can cause the mitochondrial release of AIF. Mitochondrial permeability

transition (MPT), which is a critical event of early apoptosis, results in mitochondrial swelling and leads to outer membrane rupture and thus release of pro death molecules (Wang *et al.*, 2002; Bidere *et al.*, 2003). The role of mitochondrial permeability transition in AIF release is still controversial. Therefore MPT affects AIF release or *vice-versa* is yet to be investigated.

AIF induces caspase independent cell death. Following AIF translocation, classical apoptotic features, such as phosphatidylserine exposure, partial chromatin condensation and nuclear condensation, occur in the absence of caspase activation. AIF appears to play an important role in the acute neurotoxicity induced by trauma, hypoglycemia, transient ischemia and chronic neurodegenerative diseases. AIF translocates in several experimental models of neurodegeneration, including the death of photoreceptors induced by retinal detachment, the *in vivo* neuronal death induced by brain trauma and cerebral ischemia, the death of cortical neurons induced *in vitro* by exposure to heat inactivated *Streptococcus pneumoniae,* hydrogen peroxide, peroxynitrite, the topoisomerase I inhibitor- camptothecin, and the excitotoxin NMDA. Under excitotoxic conditions, the NMDA induced mitochondrial release of AIF is PARP-1 dependent and caspase independent (Fig. 1.20) and further neutralization of AIF by an AIF specific antibody prevents cell death (Wang *et al.*, 2003; Yu *et al.*, 2002).

1.3. *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). *Dictyostelium* 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 (Fig. 1.21), 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 (Fig. 1.22). The relative proportion of prestalk cells within slugs varies between 10% to

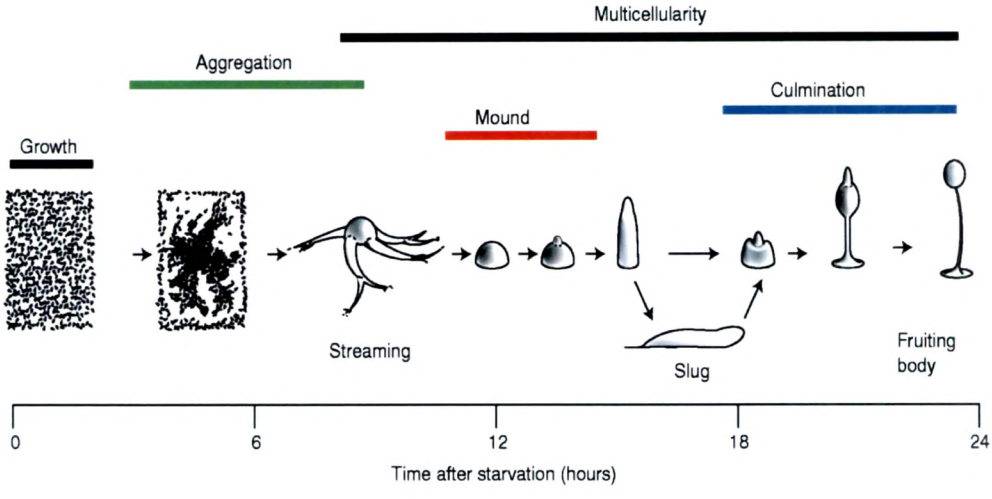


Figure 1.21: Life cycle of *D. discoideum*

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, Mcdonald 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.

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.

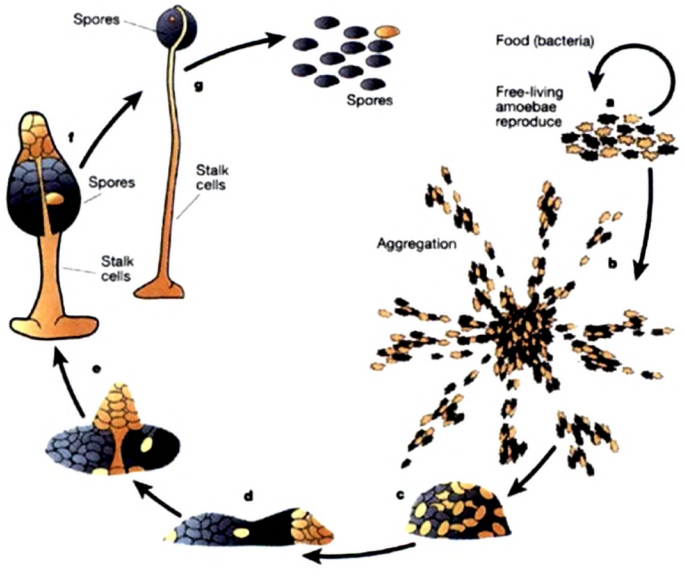


Figure 1.22: Fate of the *D. discoideum* cells during development

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.

1.3.1. 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 (Fig. 1.23).

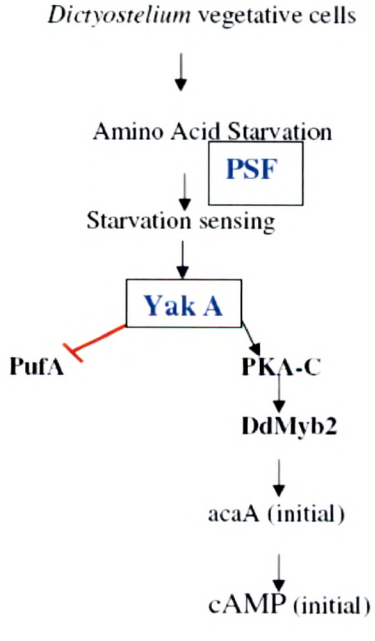
YakA mediates the initiation of development by repressing *pufA* expression. PufA, a translational regulator inhibits catalytic subunit of cAMP-dependent protein kinase A (PKA-C) translation by binding to a region at the 3'end of the *PKA-C* mRNA (Souza *et al.*, 1999). Thus, YakA acts as a regulator 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.*

Cell density sensing:

Starvation and a threshold of cell density are the two known prerequisites for the transition from growth to development. Two secreted proteins, prestarvation factor (PSF) and conditioned medium factor (CMF) are involved in sensing cell density (Burdine and Clarke, 1995). PSF, the autocrine factor is a 68 kDa glycoprotein that is secreted when cells are growing and accumulates as an indicator of the ratio of the cell density relative to the food supply. PSF helps cells determine density with respect to each other as well as the bacterial population. When the bacterial population drops, the PSF inhibition is relieved, and PSF induces genes that trigger the developmental process. Once the nutrients are depleted, PSF production declines and a second cell density sensing pathway mediated by CMF is activated. CMF, a 80 kDa glycoprotein essential for early development (Yuen *et al.,* 1991; Yuen *et al.,* 1995; Jain *et al.,* 1992) is sequestered in vegetative cells but is secreted upon starvation. A critical concentration of extracellular CMF is required for subsequent development, as it is involved in regulating aggregation, cAMP pulsing and early developmental events (Gomer *et al.,* 1991; Yuen *et al.,* 1995; Jain and Gomer, 1994).

(A)



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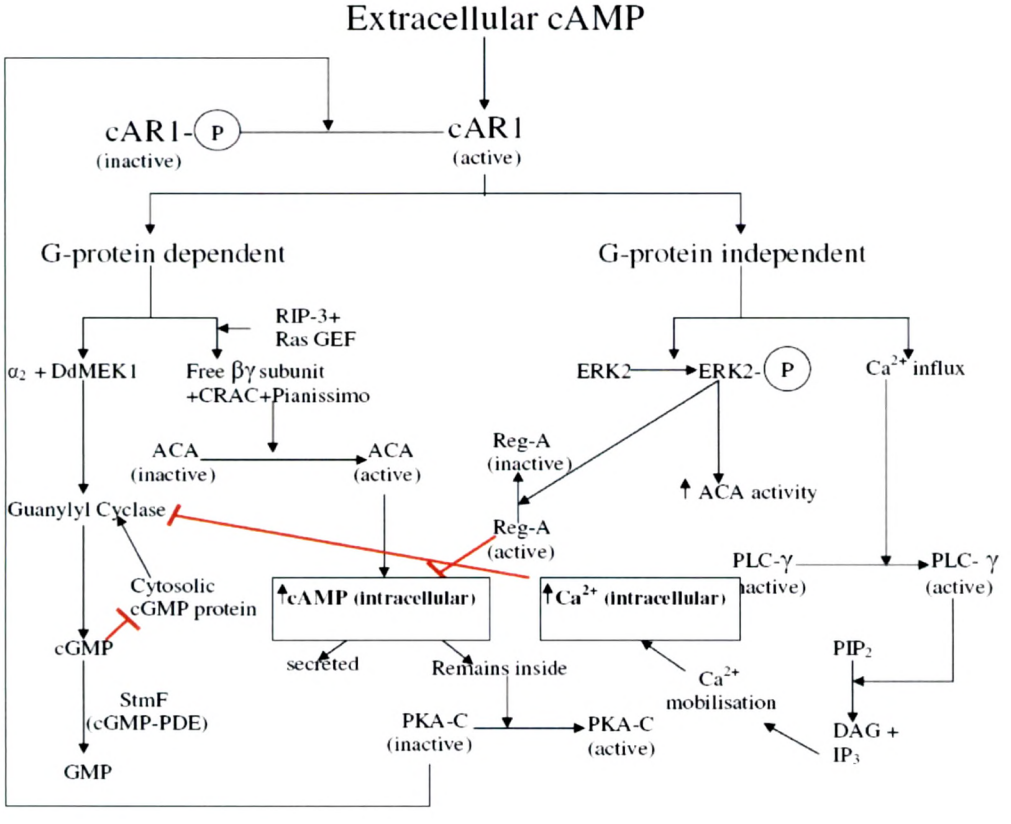

Figure 1.23: A schematic description: (A) The pathway controlling the onset of *D. discoideum* development. (B) The pathway induced by cAMP during *D. discoideum* development.

Cell size regulation:

Countin and Countin 2 proteins are thought to control the size of the multicellular structure in *D. discoideum* since the *countin*$^-$ strain forms a huge fruiting body and *countin2*$^-$ strain forms a small fruiting body. Countin is required for the proper function of a cell counting mechanism that regulates organism size in *D. discoideum*

(Brown and Firtel, 2000). A field of starving amoebae first breaks up into territories. In each territory, the cells form a spider like pattern of streams of cells. As part of a negative feedback loop, counting factor (CF), a secreted protein complex whose concentration increases with the size of the stream, prevents over sized fruiting bodies being formed by increasing cell motility and decreasing cell-cell adhesion, which causes the breakup of excessively large streams (Brock and Gomer, 1999; Brock *et al.*, 2002, 2003, 2006). Thus counting factor and countin 2 regulate group size in *D. discoideum.*

Components of the cAMP relay system:

The cells achieve competence to relay cAMP signals within a period of 6 hours. cAMP secreted by a starving cell in a pulsatile manner acts as the first messenger and activates several pathways in the cell itself as well as surrounding cells through cAR signal transduction (Sun and Devreotes, 1991). Initially at lower concentration of cAMP, the receptors undergo excitation leading to a cascade of processes, but as cAMP concentration rises extracellularly, the receptors become desensitized due to the modification/ sequestration/ internalization/ degradation of receptors/ uncoupling of receptor and target proteins etc. The reversal of desensitization is facilitated by removal of cAMP by extracellular phosphodiesterase (ePDE).

cAMP receptors:

D. discoideum also provides a powerful system for studying the role of receptors in controlling the dynamics of cell-cell signaling during multicellular development. The starvation induced chemotactic aggregation of individual cells is controlled by propagating waves of the chemoattractant cyclic AMP. (During development the cAMP signal is detected and transduced by a family of at least four cAMP receptors (cAR1-cAR4), which differ in their expression levels and pattern. The cAMP receptor types expressed sequentially during development have decreasing affinities for cAMP possibly to enable the organism to cope with an increase in extracellular cAMP concentration during the formation of the multicellular structures (Winckler *et al.*, 2004). The high affinity receptor cAR1 is the first to be expressed during early aggregation; it is the primary receptor responsible for aggregation since cells lacking cAR1 fail to aggregate. cAR1 continues to be expressed later during development in all the cells. During later

aggregation a small number of cAR3 receptors are expressed. In the slug, the expression of cAR3 becomes confined to the prespore cells (Saxe *et al.*, 1993). cAR2 is first expressed at the mound stage where it is restricted to the cells that form prestalk zone.

cAMP upon binding to cAR1 transduces the signal to a G protein complex consisting of a $\alpha_2\beta\gamma$. $\beta\gamma$ then dissociate from the complex and activate phosphatidylinositol-3 kinase (PI3K). PI3K converts phosphatidylinositol (4,5) bisphosphate (PIP2) in the plasma membrane into phosphatidylinositol (3,4,5) triphosphate (PIP3). PIP3 binds to the pleckstrin homology domain of cytosolic regulator of adenylyl cyclase (CRAC), which translocates from cytosol to membrane where it can activate adenylyl cyclase A (ACA). Positive feedback causes an increase in cAMP secretion, till cAR receptors get adapted and do not activate ACA further. Receptor phosphorylation could be the reason for receptor adaptation. The resulting drop in cAMP production combined with an active degradation mediated by phophodiesterase A (PdsA) terminates the loop. Many proteins like PdsA inhibitor (PDI), Mitogen activated protein kinase (MAPK), Ras etc. play important role in this oscillatory network.

Adenylyl cyclases:

Genes encoding three distinct adenylyl cyclases have been characterized and shown to be expressed at different stages of development in *D. discoideum*. G-protein coupled adenylyl cyclase A (ACA) produces extracellular cAMP, which is the signaling molecule required for the chemotaxis and aggregation of neighbouring cells. The osmosensory adenylyl cyclase, *acg* is expressed only during germination of spores (Pitt *et al.*, 1992; Van *et al.*, 1996). The other adenylyl cyclase gene, *acr*A encodes a protein (ACR) with domains related to histidine kinases and response regulators, while the C terminal portion contains the adenylyl cyclase catalytic domain. ACR is present at low levels in vegetative cells but accumulates dramatically following aggregation. The temporal expression of these genes may partially account for their stage specificity e.g. ACA is activated when the G-protein coupled surface receptor cAR1 binds extracellular cAMP however, during culmination, ACR activity is tenfold higher than ACA activity.

Protein Kinase A (PKA):

cAMP-dependent protein kinase (PKA) levels increase several fold during differentiation in *D. discoideum*. PKA is a heterodimer containing the single catalytic subunit PKA-C associated with a single regulatory subunit PKA-R (Mann *et al.*, 1992, Simon *et al.*, 1992). PKA is required for the regulation of early developmental genes, such as *aca* and *car*1, and plays a key role in cell-type differentiation. Cells lacking PKA-R are constitutively active for PKA, and early developmental genes are induced prematurely (Zhang *et al.*, 2003). The MAPK Erk2 is also required for ACA activation and is activated by cAMP but the mechanism is unknown (Schenk *et al.*, 2001; Segall *et al.*, 1995).

The cAMP signaling apparatus also includes polarization acquisition, actin polymerization, and cortical myosin modules, which describe the ability of a cell to create well defined anterior and posterior regions. This signaling is mediated by several MAP kinases, one such is MEK located in cytosol and translocates to the plasma membrane. Cells lacking MEK1 develop and produce cAMP normally, but respond to chemoattractant with attenuated movements (Ma *et al.*, 1997).

Induction of gene expression by cAMP pulses:

Oscillations in extracellular cAMP levels are essential for developmental initiation of *D. discoideum* cells. Strains that fail to produce cAMP pulses do not aggregate, but can be induced to do so by exogenous addition of cAMP pulses. Interestingly these strains can also be rescued by expression of the *pka*C (Wang and Kuspa, 1997). Many of the genes are induced by cAMP oscillations, like *cAR1*, *Myb2* which are implicated in *aca*A induction (Otsuka and Van Haastert, 1998) and also *dia2* (Hirata *et al.*, 2008), *countin*, *cs*A (contact site A) etc. cAMP also induces a set of five genes which code for Ca^{+2} binding proteins (*cbp*). cAMP oscillations and induction of developmental genes result in cell polarization, pseudopod formation, chemotaxis, cell - cell adhesion and further downstream events.

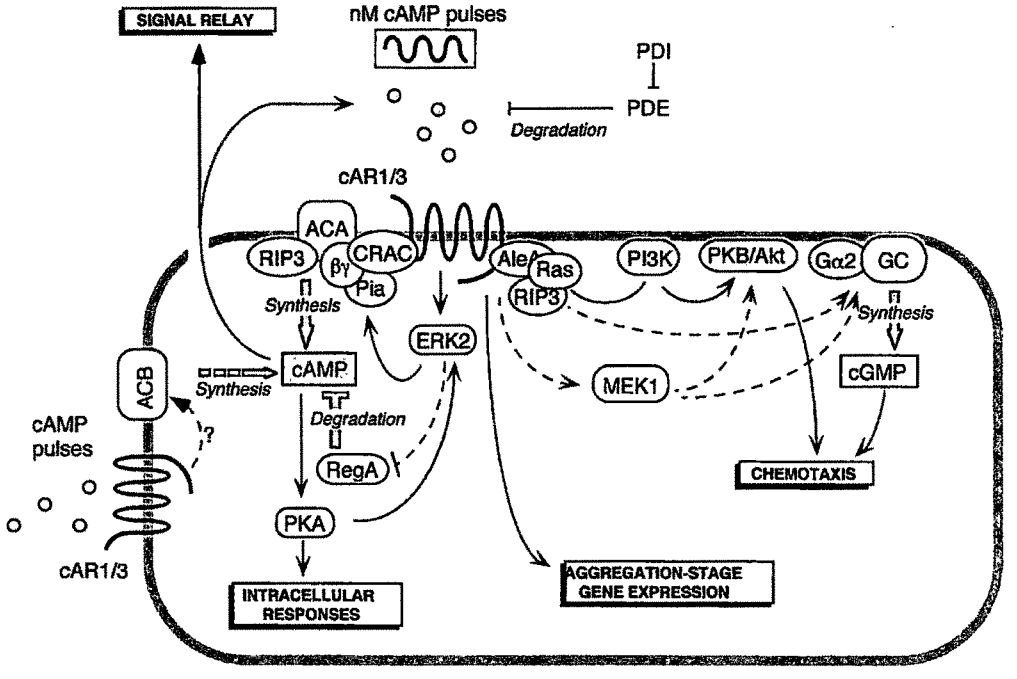

Figure 1.24: Signaling network functioning during aggregation in *D. discoideum* development

Cell differentiation:

A developmental switch occurs in response to extracellular cAMP concentration within the aggregate leading to the downregulation of the aggregation stage pathways and the activation of post aggregative gene expression (Kimmel and Firtel, 1991; Williams, 1991). This is followed by the induction of cell type differentiation. These genes include the transcription factor GBF (G-box binding factor) and the cell surface signaling molecule LagC (Dynes *et al.*, 1994; Reymond *et al.*, 1986). The cAR1 activation of GBF-mediated pathways requires micromolar concentration of cAMP and conditions in which the receptor is fully saturated.

1.3.2. *D. discoideum* as a model organism

Because of the simple life cycle of *D. discoideum*, it is commonly used as a model organism to study various aspect of the cell. One of the incredible strengths of this model system is the capacity to track the dynamic behavior of individual cells, yet can

function as a collection of cells. This makes *D. discoideum* the best of both the worlds – an easily studied single cell organism for one half of its life cycle, and a multicellular creature during the other half, such that it can be used to understand the fundamental principles of cell behavior. It can be observed at organismic, cellular, and molecular levels primarily because of their restricted number of cell types, behavior, and rapid growth. The genome repertoire of *D. discoideum* allows the expression of features like cell type determination, spatial patterning, chemotaxis, altruistic cell death and other fundamental aspects essential in multicellular organisms (Eichinger and Noegel, 2003; Ennis *et al.*, 2003). It is also used to study other aspects of development including cell sorting, pattern formation, phagocytosis, motility, and signal transduction. These processes and aspects of development are either absent or too difficult to view in other model organisms. *D. discoideum* is closely related to higher metazoans. It carries similar genes and pathways making it a good candidate for gene knockout studies (Landree and Devreotes, 2004). *D. discoideum* makes a valuable model also because of a small genome (approx. 3.7 X 10^7 bp) compared to the higher eukaryotes. The *D. discoideum* genome is approximately 40 Mbp, with approximately 10,000–13,000 genes on six chromosomes. Genome haploidy makes it relatively easy to generate and select mutants. Advantages of *D. discoideum* as a model organism include its genetic, biochemical, and cell biological accessibility. Genetic tools such as gene targeting by homologous recombination; structure function analysis by random mutagenesis; insertional mutagenesis, which allows easy cloning of the gene disrupted; and multicopy suppression libraries are available (Landree and Devreotes, 2003). The vegetative growth and developmental forms in *D. discoideum* are temporally separated; the developmental mutants can be propagated under vegetative conditions thus behaving like conditional mutants. The discoveries made in this model organism will provide insights into the working of other organisms.

***D. discoideum* is used to study the following processes:**

Cell Differentiation:

Coordinated cell type differentiation and morphogenesis lead to a final fruiting body in *D. discoideum* that allows the dispersal of spores. The study of these processes is having increasing impact on our understanding of general developmental mechanisms.

The availability of biochemical and molecular genetics techniques has allowed the discovery of complex signaling networks which are essential for *D. discoideum* development and are also conserved in other organisms. Developmental and cell type specific gene expression and differentiation have been extensively characterized in this model which can be implicated in higher eukaryotes.

Chemotaxis:

Chemotaxis is an oriented movement of a cell in response to a chemical gradient. Many cell types exhibit this phenomenon like, bacteria and many amoebae can move in the direction of food source. In our body immune cells like macrophages and neutrophils can move towards invading cells. Other cells, connected with the immune response and wound healing, are attracted to areas of inflammation by chemical signals. In addition, many of the morphogenetic (shape altering) movements that occur during the course of development are associated with cell migration in response to chemical gradients. *D. discoideum* is a beautiful system to study chemotaxis as during starvation these cells display chemotaxis towards chemoattractant- cAMP (Aubry and Firtel, 1999).

Signal transduction:

A detailed understanding of signaling system in *D. discoideum* provides clues to the mechanisms of intercellular communication in the development of metazoans. There are various signaling pathways involved in chemotaxis, cell-cell interaction, cell proportioning and differentiation. Because many of the proteins and mechanisms are conserved in higher organisms, it is not surprising that *D. discoideum* is leading the way in the discovery of the key events in chemoattractant signaling (Aubry and Firtel, 1999).

Phagocytosis:

D. discoideum shows lectin type receptor specific, and non receptor mediated phagocytosis (Vogel *et al.*, 1980). *D. discoideum* is a popular model system for studying phagocytosis as many of the similar intracellular signaling molecules and regulators do appear in mammalian system (Bush *et al.*, 1996; Harris *et al.*, 2001).

Host model for pathogenesis:

D. discoideum can also be used as a host to study various infection processes such as amoebic dysentery, amoebic keratitis, granulomatous amoebic encephalitis (GAE), and primary amoebic meningoencephalitis caused by amoebae. *Legionella* is a genus of bacteria that includes species that can cause Legionnaire's disease in humans. *D. discoideum* is also a host for *Legionella* and is a suitable model for studying the infection process (Solomon *et al.*, 2000). Specifically, *D. discoideum* shares with mammalian host cells, a similar cytoskeleton and cellular processes relevant to *Legionella* infection, including phagocytosis, membrane trafficking, endocytosis, vesicle sorting and chemotaxis (Lu and Clarke, 2005).

1.3.3. *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). *Dictyostelium* 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.

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