2. LITERATURE REVIEWS

1. Shrimp disease and defense mechanism

1.1 Penaeid shrimp diseases

Shrimp aquaculture is an important component in the economy of inter-tropical countries in Southeast Asia, Central America and South America. In 1991 and 1992, over 700,000 metric tons (mt) of cultured shrimp were produced worldwide, with 80% of production coming from Asia. Global shrimp production in 1993 was 639,000 mt, about 12% lower than 1992 production. Crop failure in China in the summer of 1993 greatly affected the world's farmed shrimp production. The explanation for these crop failures was the occurrence of infectious diseases (Rosenberry, 1994). Diseases caused by viruses, bacteria, fungi and protozoa are considered very significant to shrimp culture. Several outbreaks of shrimp diseases have caused major problems in countries such as China, Thailand, Indonesia, Taiwan, and Ecuador (Gabriel and Felipe, 2000).

1.2 White spot syndrome virus and related researches on white spot syndrome virus

White spot syndrome virus (WSSV) is the most serious viral disease of cultured shrimp in the world, and it causes large economic losses to the shrimp farming industry (Lightner, 1996). In addition to the economic impact of the disease, the natural marine ecology is also threatened because WSSV is able to infect a large number of crustaceans including crabs and crayfish (Lo et al., 1996a; Wang et al.,

1998a). WSSV was first discovered in the Chinese Province of Fujian in 1992. It quickly spread to other areas in Southeast Asia and subsequently to the global shrimp farming areas (Cai et al., 1995; Flegel, 1997). WSSV was reported in the United States in 1995 (Rosenberry, 1996) and from Central America and South America in early 1996 (Rosenberry, 2000). In 2002, WSSV was detected in Europe (France) and the Middle-East (Iran) (Rosenberry, 2002).

This virus has infected a number of other crustacean species such as amphipods, ostracods, swimming crabs, crayfish, and copepods. In the case of shrimp, WSSV infects and causes disease in many species of shrimp worldwide, including *P. monodon*, *P. semisulcatus*, *P. merguiensis*, *P. indicus*, *P. chinensis*, *P. penicillatus,* and *P. japonicus*. Clinical signs of the disease are a rapid reduction in food consumption, lethargy, a loose cuticle, a pink to reddish-brown discoloration and white spots on the carapace of the cephalothorax (Chou et al., 1995; Lightner, 1996). The virus severely damages the stomach, gills, subcuticular epithelial cells, lymphoid organ, antennal gland, and haemocyte (Chang et al., 1996; Lightner, 1996).

WSSV is a rod-shaped DNA virus belonging to the virus family *Nimaviridae*, genus *Whispovirus* (Mayo, 2002) that causes 100% mortality of cultured shrimps in 3-4 days (Lightner, 1996). These viruses are enveloped and have a bacilliform morphology measuring 266±13 nm in length and 112±7 nm in width. The nucleocapsid measures 420±18 nm in length and 68±5 nm in width (Sahul-Hameed et al., 1998). The double-stranded viral DNA is 305 kb with 181 open reading frames (ORFs) (van Hulten et al., 2001; Yang et al., 2001). WSSV has been isolated and characterized from the Indian shrimp, *P. indicus* by Sahul-Hameed et al. (1998) and was found to be similar to the WSSV, as described by Wongteerasupaya et al. (1995).

However, sequence analysis by various authors has revealed variations among different WSSV isolates (Lo et al., 1999; Wang et al., 2000a). One Chinese WSSV genome isolate listed at Genbank (accession no. AF332093) has a genome of 305 kb dsDNA (Yang et al., 2001), and another from *P. monodon* in Thailand has a 293 kb genome (Genbank accession no. AF369029) (van Hulten et al., 2001). Huang et al. (2002) were able to identify 18 proteins of WSSV by one-demensional SDS-PAGE followed by mass spectrometry and transcriptional analysis, and major virion protein genes (vp28, vp26, vp24, vp19, and vp15) have been identified (Marks et al., 2003).

Because of the devastation effects on shrimp farms by WSSV various diagnostic methods with high efficiency, simplicity of use, and accuracy have been developed in many different laboratories throughout the world to help monitor and control the spread of WSSV. Several diagnostic methods have been described, such as Polymerase Chain Reaction (PCR) (Kimura et al., 1996; Lo et al., 1996b; Nunan and Lightner, 1997; Kasornchandra et al., 1998; Kim et al., 1998; Tapay et al., 1999; Kaitpathomchai et al., 2001; Yoganandhan et al., 2003; Galaviz-Silva et al., 2004), in situ hybridization (Chang et al., 1996; Durand et al., 1996; Wongteerasupaya et al., 1996; Wang et al., 1998b), miniarray (Quere et al., 2002), observation of tissues subjected to fixation or negative staining (Inouye et al., 1993), detection of virus in the hemolymph or gastric epithelium by dark field microscopy (Momoyama et al., 1995) and immunological methods using monoclonal and polyclonal antibodies to WSSV or their component proteins (Huang et al., 1995; Nadala et al., 1997; Sahul-Hameed et al., 1998; Nadala and Loh, 2000; Poulos et al., 2001). Recently, Reverse Passive Latex Agglutination (RPLA), a new method, has been developed. This method was used for detecting WSSV in the hemolymph of infected *P. indicus* (Sathish et al., 2004) and *P.*

japonicus (Okumura et al., 2005). RT-PCR and Western blot assay for vp19 were used to study the differential viral expression in different tissues of infected shrimp (Rout et al., 2005).

1.3 Shrimp immune response

The crustacean immune response can be divided into cellular and humoral components. The cellular component is related to haemocytes, which involved in the immediate defensive reactions such as nodulation, encapsulation and phagocytosis. The humoral component is characterized by temporarily enhanced antimicrobial activity in the cell-free hemolymph, which the clotting cascade, the synthesis of a wide array of antimicrobial peptides, and the phenoloxidase (PO)-activating system (Soderhall et al., 1992; Niere et al., 1999). Recently, several genes involved in immune response in *P. monodon* have been cloned and characterized; such as prophenoloxidase (Sritunyalucksana et al., 1999), the hemolymph clotting protein (Yeh et al., 1999), peroxinectin (Sritunyalucksana et al., 2001), and ß-1,3-glucan binding protein (GBP) (Sritunyalucksana et al., 2002).

Crustaceans have an open circulatory system with not equivalent to vertebrate red blood cells, but are analogues of the white blood cells. By morphological criteria and by different staining techniques, hemocytes can be divided into three subpopulations: (1) hyaline, (2) semi-granular and (3) granular cells (Holmblad and Soderhall, 1999, Soderhall and Cerenius, 1992).

1. The hyaline cells are characterized by the absence of granules, and are the smallest size and the least numerous haemocytes. The hyaline cells are capable of phagocytosis and cell spreading. The number of hyaline cells appears to vary in

different crustaceans. For instance, approximately 10%, 60% and 3% of the circulating haemocytes were found in *P. japonicus*, *Carcinus meanus*, and crayfish, respectively (Holmblad and Soderhall, 1999; Soderhall and Cerenius, 1992).

2. The semigranular cell contains small granules. It appears to be the first sensitive cell type in immune response when reacting with microbial polysaccharides; such as lipopolysaccharides and β-1,3 glucan (Soderhall and Cerenius, 1992). These cells also are involved in recognition and releasing of prophenoloxidase (ProPO) activating system. In addition, semi-granular cell is responsible for the encapsulation and also displays some phagocytic capacities (Bachere et al., 1995).

3. Granular cells contain a large number of secretory granules containing components of the ProPO system which is an important component of the cellular defense reactions. Once β-1,3 glucan binding protein (βGBP) has reacted with β-1,3 glucans, this protein complex becomes activate and can bind to a specific membrane receptor on granular cells. The βGBP-glucan complex induces ProPO system releasing by degranulation of granular cells (Bachere et al., 1995b; Holmblad and Soderhall, 1999; Soderhall and Cerenius, 1992).

1.4 Shrimp apoptosis

Outbreaks of white spot syndrome virus in cultured penaeid shrimp have resulted in mass mortality and serious economic loss worldwide (Flegel, 2001). It has been suggested that apoptosis induced by WSSV may be a part of the pathophysiology leading to shrimp death (Flegel and Pasharawipes, 1998; Flegel, 2001). The occurrence of apoptosis has been supported by data obtained using TUNEL, and by DAPI-staining in haemocytes and tissue. The reduction in total haemocyte count has

been observed in *P. monodon* (Wongprasert et al., 2003) and *P. japonicus* after WSSV injection (Henning et al., 1998). The total haemocyte count in *P. monodon* also decreased rapidly after infection with yellow head virus, and a large fraction of those haemocytes remaining were apoptotic (Khanobdee et al., 2002). Wongprasert et al. (2003) reported that about 20% of the haemocytes possessed condensed and fragmented nuclei, which was observed using DAPI-staining, suggesting that these haemocytes were undergoing apoptosis. Apoptosis also occurred in haematopoietic tissue. The reason for the decrease in haemoctyes in WSSV infected shrimp could be both haematopoietic tissue (Hose et al., 1987) and haemocytes themselves (Wonprasert et al., 2003).

Wang and coworker (2000b) reported that WSSV induced apoptosis in lymphoid organs observed by TUNEL technique. Sahtout et al. (2001) found the highest number (46%) of TUNEL-positive cells in the epithelium of naturally infected *P. monodon*. However, Wongprasert et al., 2003 found only 10% TUNEL-positive cells in the subcuticular epithelium of WSSV infected *P. monodon*. These differences could be due to differences in the tissue sites, the time of infection, the infection dose, or the infection route. In addition, caspase-3 activity in WSSV-infected shrimp was about 6-fold higher than that in uninfected shrimp. The increase in caspase-3 activity in WSSV infected tissue identified supports the argument that apoptosis is involved in the shrimp response to virus and it is important in the pathophysiology of WSSV infection. The data strongly suggests that apoptosis occurs following WSSV infection in *P. monodon* and apoptosis could be used in shrimp as a protective response to eliminate viral pathogens.

2. Apoptosis

Apoptosis is one type of programmed cell death characterized by a particular pattern of morphologic changes. In all organisms, cells die for a variety of reasons, both intentional and unintentional. For example, during the early stages of development in mammals, an intricate program of cell proliferation and death is required to create organs of normal size and functional ability. This also applies to the generation of an immune system that only recognizes foreign antigens and not those that are "self". Unintentional cellular insults may also trigger cell death such as those caused by ultraviolet light or chemical agents. This ordered destruction of a cell is referred to as programmed cell death or apoptosis and it is distinguishable from death by necrosis which is a considered a random event (Pollard and Earnshaw, 2002; Danial and Korsmeyer, 2004).

In contrast to necrosis, apoptosis involves cell shrinkage rather than cell swelling (Table 1 and Figure 1). Apoptosis is characterized by a reproducible pattern of structural alterations of both the nucleus and cytoplasm. In order of appearance these include; first, the cytoplasm begins to shrink following the cleavage of lamins and actin filaments. Nuclear condensation can also be observed following the breakdown of chromatin and nuclear structural proteins, and in many cases the nuclei of apoptotic cells take on a "horse-shoe" like appearance (Figure 2A). Second, cells continue to shrink (Figure 2B). Third, cells pack themselves into a form that allows for easy clearance by macrophages. These phagocytotic cells are responsible for removing apoptotic cells from tissues in a clean and tidy fashion that avoids many of the problems associated with necrotic cell death. In order to promote their phagocytosis by macrophages, apoptotic cells often undergo plasma membrane changes that trigger the

macrophage response. One such change is the translocation of phosphatidylserine from the inner leaflet of the cell to the outer surface. Membrane changes can often be observed morphologically through the appearance of membrane blebs or blisters (Figure 2C). Finally, membrane continues to change which often appears towards the end of the apoptotic process. Small vesicles called apoptotic bodies are also sometimes observed (Figure 2D, arrow) (Pollard and Earnshaw, 2002).

The apoptotic pathway and the engulfment process are part of a continuum that helps ensure the non-inflammatory nature of this death paradigm. In *Caenorhabditis elegans*, phagocytosis can help promote cell killing and an intact engulfment process requires *ced-3* (*C. elegans* death-3) (Hoeppner et al., 2001; Reddien et al., 2001). The cast orchestrating the clearance of apoptotic cell bodies in nematodes consists of at least seven genes, which have homologs in higher organisms. These genes were further divided into two partially redundant classes and the most dramatic engulfment defects were seen when one gene from each category was altered in double-mutant animals (Ellis et., 1991). *ced-1* encodes an engulfment receptor (Zhou et al., 2001), *ced-6* which is homologous to the mammalian PTB domain-bearing adaptor GULP (Liu and Hengartner, 1998) and *ced-7* which encodes a protein with homology to ABC-1 transporter (Wu and Horvitz, 1998a) belong in one category and help recognize apoptotic cells. *ced-2* (CrkII) (Reddien and Horvitz, 2000), *ced-5* (DOCK-180) (Wu and Horvitz, 1998b), *ced-10* (small GTPase Rac-1) (Reddien and Horvitz, 2000), and *ced-12* (ELMO) (Gumienny et al., 2001) constitute the second class of genes and influence cytoskeletal remodeling.

Phagocytes recognize the surface of the dying cell probably through an "eat me" signal. In mammalian systems, the best characterized "eat me" signal is

phosphatidylserine (PS) displayed on the plasma membrane of dying cells (Fadok et al., 2000). Evidence has been marshaled for the participation of multiple engulfment receptors including CD91, CD14, CD36, and $\alpha_V\beta_3$ interin, and the phosphatidylserine receptor (PSR) (Figure 3) (Savill and Fadok, 2000).

The disposal of the apoptotic corpse is plotted once "eat me" signals on its surface are engaged by engulfment receptors. In *C. elegans,* the receptor encoded by *ced-1* clusters around the dying cell in a manner that utilizes *Ced-7* (Zhou et al., 2001). Interestingly, ABC-1, the ortholog of CED-7, is believed to regulate the distribution of PS in the membrane (Hamon et al., 2000). *ced-7* is unique among cell engulfment genes because it functions both in phagocytes and apoptotic cells (Wu and Horvitz, 1998a). Binding of engulfment receptors to apoptotic cells ultimately signals cytoskeletal events. An interaction between the CED-1 cytoplasmic tail and CED-6 (Su et al., 2002) may serve this role consistent with genetic studies ordering *ced-1* upstream of *ced-6* (Liu and Hengartner, 1998).

Studies in mammals have highlighted the importance of proper disposal of corpses by phagocytic cells (Savill and Fadok, 2000). In addition to engulfment of apoptotic cells, macrophages are important regulators of proinflammatory responses by releasing cytokines such as TNFα. While proinflammatory factors are necessary in immune reaction against infection, their suppression during apoptotic corpse clearance is essential. This is partially accomplished by release of anti-inflammatory factors including TGFß and IL-10 by macrophages engaged in corpse engulfment. Furthermore, regulatory mechanisms help ensure that when phagocytosing dendritic cells present peptides from apoptotic corpses to T cells, no immune reaction against

self peptides is initiated. Defects in clearance of corpses are predicted to create a proinflammatory milieu that may predispose to autoimmune disorders.

Since the concept of apoptosis was established in 1972 (Kerr et al., 1972) research effects have led to the identification of hundreds of genes that control the initiation, execution, and regulation of apoptosis in several species (Danial and Korsmeyer, 2004). Compelling evidence shows that the mechanism of apoptosis is evolutionarily conserved.

2.1 The mechanisms of apoptosis

Apoptosis is a distinct form of cell death that proceeds along a genetically determined execution program. It exhibits a characteristic morphology (Majno and Joris, 1995) and features unique biochemical alterations (Enari et al., 1998). An increasing number of genes involved in the execution of the apoptosis program is identified and concepts of different, although interacting, apoptosis signaling pathways are delineated (Figure 4) (Ashkenazi and Dixit, 1998; Green, 1998; Vaux and Korsmeyer, 1999). There are 3 different mechanisms by which a cell commits suicide by apoptosis (Mullauer ey al., 2001).

Table 1. Comparison of apoptosis and necrosis.

| Apoptosis	Necrosis
General	
Affects isolated cells	Affects clusters in cells
No inflammatory response	Inflammatory cells invade tissue
Metabolic	
Early increase in protein & RNA	Switching off protein synthesis synthesis and phosphorylation
Cell membrane remains intact	Cell membrane becomes leaky
Affected by protein & RNA Synthesis inhibitors	
Endonuclease activation (calcium- & Magnesium-dependent)	
ATP-dependent	ATP-depleted
Morphological	
Loss of cell contact, early	Loss of cell contact, late
Shrinkage of cell	Swelling of cell (sodium ions and water taken up by cell)
Membranes remain intact (no enzyme leakage)	Membrane defects (internal & external) (enzyme leak out)
Condensation of cytoplasm	Cytoplasm becomes clear
	Mitochondria swell and acquire dense bodies.
Condensation and margination of chromatin	Lysosomes disrupted (release of enzymes)
Sharp edges to chromatin masses	Irregular edges to chromatin masses.
Nuclear fragmentation	No fragmentation
Cytoplasmic budding and fragmentation	
Phagocytic removal	

Potten and Wilson (2004)
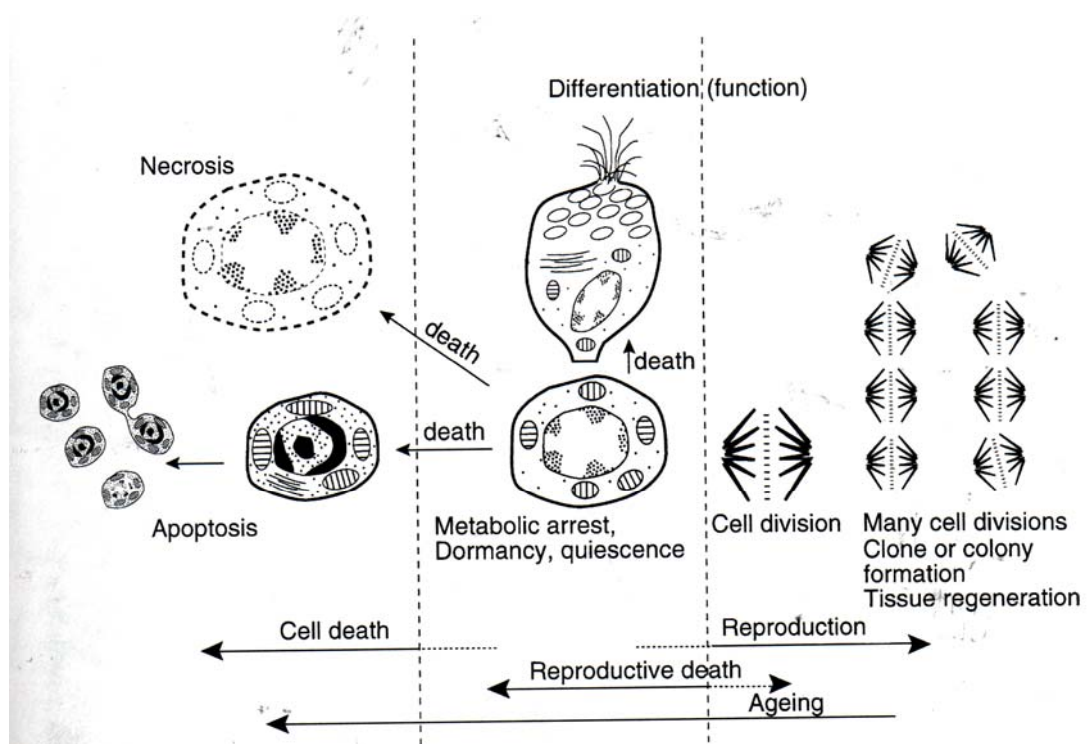
Figure 1. Diagrammatic representation of cell fate, showing commonly observed morphological features. (Potten and Wilson, 2004)
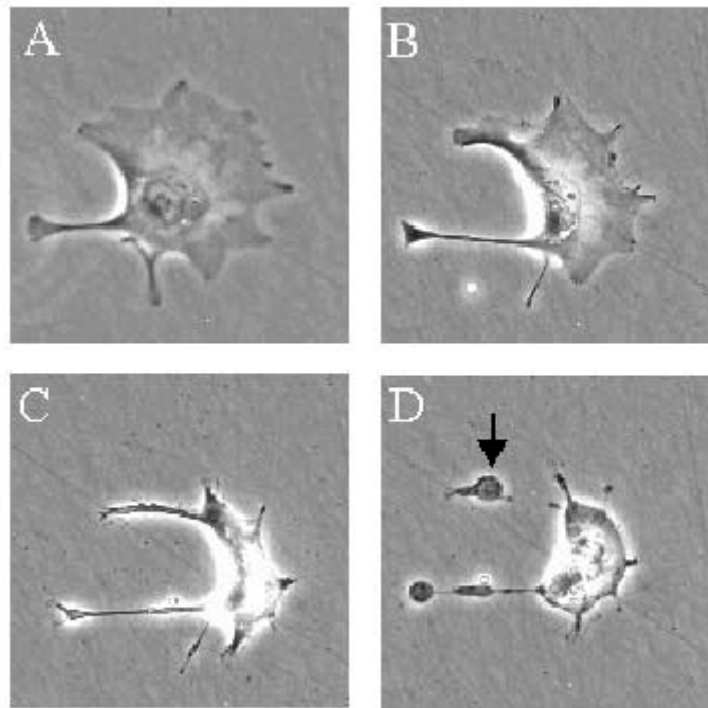
Figure 2. Apoptosis process.

A, Nucleus breaks down. *B,* Cell shrinks in size. *C,* Signal to macrophage through plasma membrane changes. *D,* Signal through apoptotic bodies.
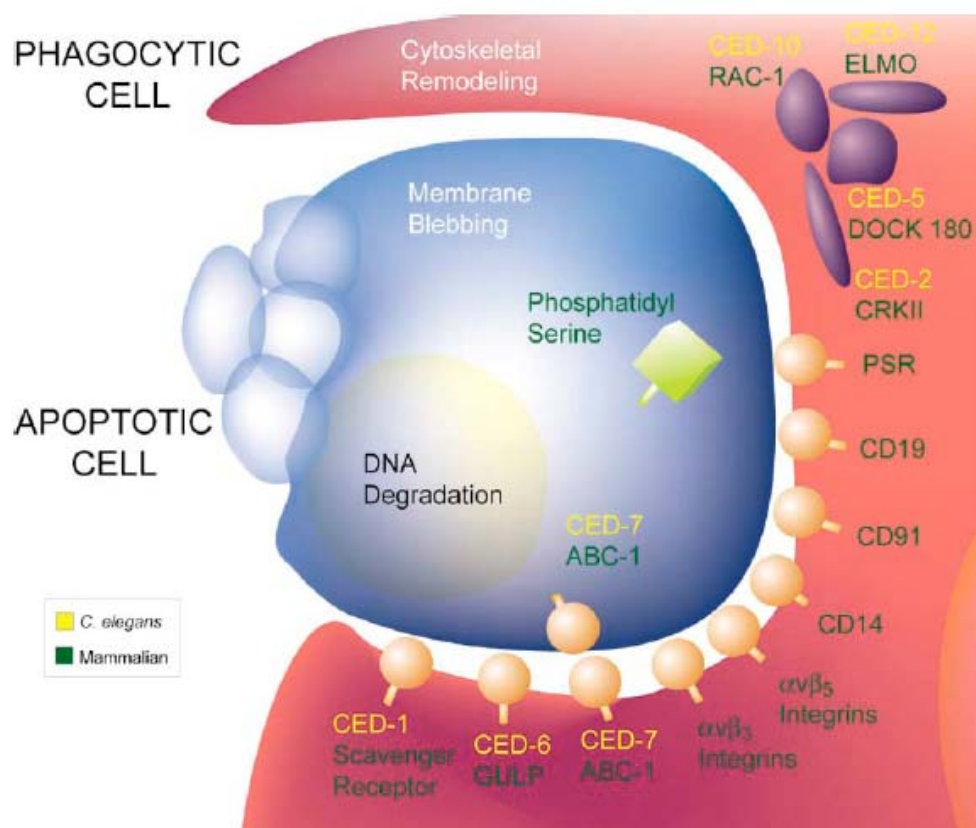
Figure 3. Engulfment of apoptotic cells.

The engulfment machinery in mammals and *C. elegans* share evolutionarily conserved elements. Proteins encoded by two partially redundant categories of genes in *C. elegans* involved in this process are labeled in yellow and their mammalian counterparts are labeled in green (Danial and Korsmeyer, 2004).

2.1.1 Apoptosis triggered by internal signal: the intrinsic or mitochondrial pathway

A first major apoptosis pathway involves mitochondria. A key molecule in mitochondrial cell death is cytochrome c. When released from mitochondria in response to cell damage, it binds to the cytoplasmic adaptor molecule apoptotic protease-activity factor-1 (Apaf-1) (Green and Reed, 1998; Bossy-Wetzel and Green, 1999; Kroemer and Reed, 2000). The Apaf-1 then recruits pro-caspase 9, which becomes activated by autoprocessing and triggers a caspade of downstream caspase reactions (Figure 4). Members of the Bcl-2 family are involved in the regulation of mitochondrial cell death (Adams and Cory, 1998; Reed, 1998; Gross et al., 1999). Anti-apoptotic members like Bcl-2 and Bcl-$_{XL}$ inhibit cytochrome c release from mitochondria (Kluck et al., 1997; Yang et al, 1997). Pro-apoptotic members like Bax may act by forming pore complexes in the outer mitochondrial membrane (Schendel et al., 1998).

2.1.2 Apoptosis triggered by an external signals: the extrinsic or death receptor pathway

A second apoptosis pathway involves cell surface "death receptors" (DR) that transmit an apoptosis signal on binding of a specific "death ligand" (Ashkenazi and Dixit, 1998). The largest known family of DRs is represented by tumor necrosis factor receptors (TNF-Rs) (Baker and Reddy, 1998). The best characterized members are TNF-R1 (also called p55, CD120a), TNF-R2 (p75, CD120b), Fas (CD95, Apo-1), death receptor 3 (DR3), death receptor 4 (DR4, TRAIL-R1) and death receptors 5 (DR5, TRAIL-R2) (Figure 4 and 5) (Ashkenazi and Dixit, 1998; Mullauer et al., 2001;

Danial and Korsmeyer, 2004). The ligands that activate these receptors are structurally related molecules with homologies to tumor necrosis factor α (TNFα) (Ashkenazi and Dixit, 1998; Griffith and Lynch, 1998; Ledgerwood et al., 1999; Nagata, 1999; Pinkoski and Green, 1999). Upon ligand binding an intracellular "death domain" of the receptor interacts with a homologous domain in an adaptor protein, which recruits specific proteases, the so-called caspases (Figure 4 and 5). Caspases (for "cysteine aspartase") are cysteine dependent proteases that exert a limited proteolyses by cleavage of their substrate after specific aspartate residues. They reside as inactive pro-forms within the cell and become activated by autocleavage when recruited to a DR signaling complex. Activated upstream caspases subsequently initiate a cascade of downstream effector caspases which cleave a plethora of cellular proteins and; thereby, ultimately cause cell death (Thornberry and Lazebnik, 1998; Kumar, 1999; Slee et al., 1999).

2.1.3 Nuclear protein, p53

A third major pathway is represented by the nuclear protein p53 (Levine, 1997; Amundson et al., 1998; Prives and Hall, 1999; Sionov and Haupt, 1999). The p53 is activated in response to DNA damage. It blocks cells with damaged DNA in the G1 and G2 phase of the cell cycle (Bunz et al., 1998). If the DNA damaged is serve, and dependent on cell type and oncogene composition of a cell, p53 initiates apoptosis by mechanisms that partially rely on the transcription of apoptosis executionary genes like Bax (Reed, 1998) and genes whose products generate reactive oxygen species (Polyak et al., 1997).

p53 is implicated in the induction of what had until recently been understood to be two distinct apoptotic signaling pathways that lead to the activation of the aspartate-specific cysteine proteases (caspases) that mediate apoptosis (Figure 6). The extrinsic pathway involves engagement of particular 'death' receptors that belong to the tumor necrosis factor receptor (TNF-R) family and, through the formation of the death-inducing-signaling-complex (DISC) (Ashkenazi and Dixit, 1998), leads to a cascade of activation of caspases, including caspase-8 and caspase-3, which in turn induce apoptosis. The intrinsic pathway is triggered in response to DNA damage and is associated with mitochondrial depolarization and release of cytochrome c from the mitochondrial intermembrane space into the cytoplasm. Cytochrome c, apoptotic protease-activating factor 1 (Apaf-1) and procaspase-9 then form a complex termed the apoptosome, in which caspase-9 is activated and promotes activation of caspase-3, caspase-6 and caspase-7 (Nicholson and Thornberry, 2003). Recent studies; however, link the extrinsic and intrinsic pathways, lending support to the idea of converging rather than distinct pathways (Li et al., 1998; Gross et al., 1999).
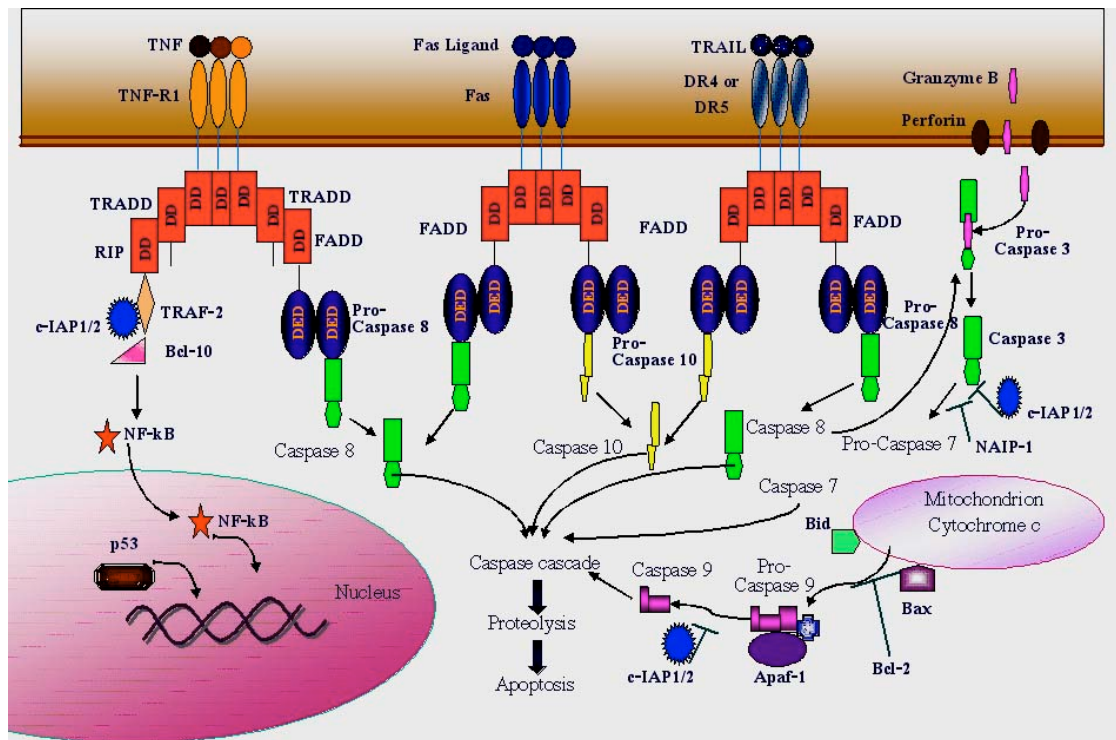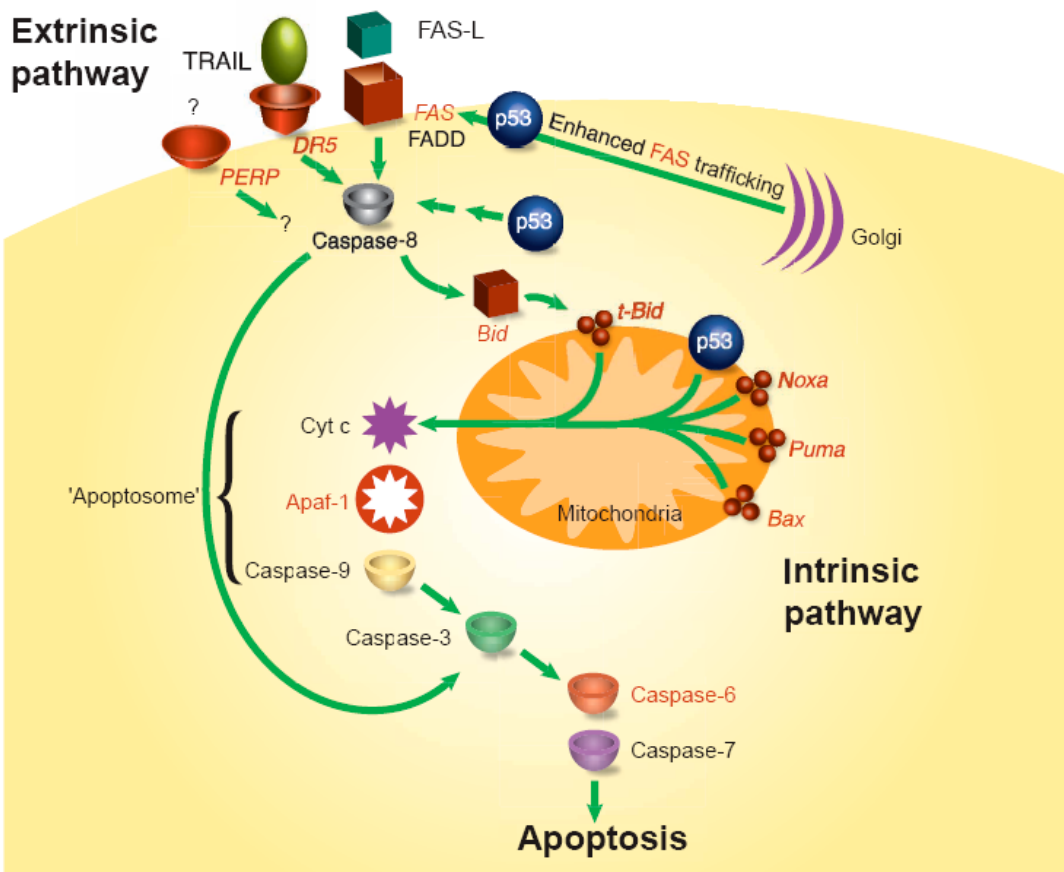
Figure 4. Simplified scheme of major apoptosis pathway.

DD: death domain, DED: death effector domain. (Mullauer et al., 2001)
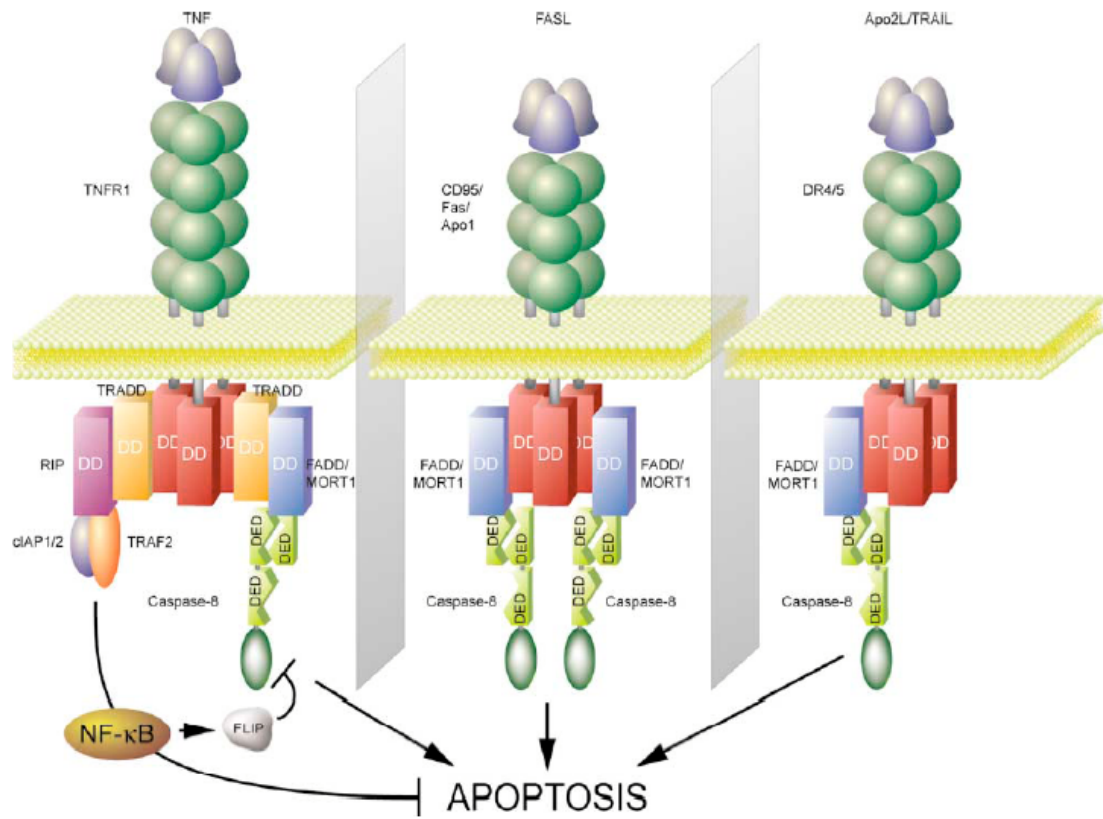
Figure 5. Extrinsic death receptor pathway.

The distinct composition of the Death-Inducing-Signaling Complex (DISC) downstream of the various death receptors TNFR1, CD95, and DR4/5 is illustrated. (Danial and Korsmeyer, 2004)

Figure 6. A model for p53-mediated apoptosis.

This model depicts the involvement of p53 in the extrinsic and intrinsic apoptotic pathways. p53 target genes are shown in red. The convergence of the two pathways through Bid is shown. (Haupt et al., 2003)

2.2 Proteins involved in apoptosis

2.2.1 Bcl-2 family

There are many proteins that regulate apoptosis and their levels may go up or down, or their state of activation or cellular localization may change during apoptosis. These include members of the B-cell lymphoma leukemia-2 (Bcl-2) family of proteins as shown in Table 2, which are named after the first member to be discovered (Bcl is an acronym for B-cell lymphoma), and the protein p53, which orchestrates the response of the cell to DNA damage (Adams and Cory, 1998; Chao and Korsmeyer, 1998; Pollard and Earnshaw, 2002). Many cell-based studies have demonstrated that overexpression of Bcl-2 enhances resistance to apoptosis, and the oncogenic potential of Bcl-2 has been confirmed in mouse models. Transgenic mice overexpressing Bcl-2 in the B-cell compartment develop B-lymphoid tumours (Adams et al., 1999). Bcl-2-related proteins can act as cellular bodyguards or assassins to positively or negatively control apoptosis (Cory et al., 2003; Green and Kroemer, 2004; Kirkin et al., 2004). Each member of the Bcl-2 family possesses at least one of four Bcl-2 homology domains (BH; BH1 to BH4) (less than 20 amino acid residues in length). More than 20 family members have been identified; and these can be divided into two subgroups, multi-domain (three or four BH domains) and BH3-only Bcl-2 family proteins (Figure 7). The group of multi-domain Bcl-2 family proteins consists of both pro-apoptotic; such as Bax and Bak, and anti-apoptotic, such as Bcl-2 and Bcl-x_L, whereas BH3-only proteins are proapoptotic, such as Bad and Bid (Figure 7) (Huang, 2002; Igaki and Miura, 2004).

In mammalian cells, pro- and anti-apoptotic members of the Bcl-2 family act as molecular determinants for cell death decisions. Genetic and biochemical studies in

the nematode *C. elegans* have revealed that worm cell death is also regulated by two opposing members of the Bcl-2 family, (1) EGL-1 and (2) CED-3, CED-4, CED-9. The *ced-3* gene product is a homologue of mammalian interleukin-1ß-convering enzyme (ICE), which is a member of a unique cysteine protease family, the caspase. CED-4 is an adaptor protein homologous to apoptotic protease activating factor-1 (Apaf-1). CED-9 is an ortholog of the anti-apoptosis members of the Bcl-2 family. Genetic studies have shown that the *ced-9* gene is involved in preventing cell death by repressing *ced-3* and *ced-4* activities (Figure 8) (Hengartner et al., 1992; Igaki and Miura, 2004).

In Drosophila, as well as in mammals, cell death is essential for normal embryogenesis and organ development, especially in the central nervous system, and is also important for metamorphosis. Similar to the mammalian cell death machinery, Drosophila possesses a more-complicated cell-death regulatory system than do nematodes: the fly has an Apaf-1-like protein (Dark/Dapaf-1/HAC1) (Kanuka et al., 1999; Rodriguez et al., 1999; Zhou et al., 1999), seven caspases (Fraser and Evan, 1997; Chen et al., 1998), and two Bcl-2 family members (Figure 8) (Colussi et al., 2000; Brachmann et al., 2000; Igaki et al., 2000; Zhang et al., 2000). Drosophila also has unique killer proteins, Reaper, Hid, and Grim (White et al., 1994; Grether et al., 1995; Chen et al., 1996). A deficiency of all three of these proteins eliminates all the cell death during embryogenesis, resulting in embryonic lethality (White et al., 1994). Genetic studies have demonstrated that Dark and the caspases function downstream of Reaper, Hid, and Grim, and that the overexpression of any of these proteins is sufficient to induce cell death. The activities of Reaper and Grim are regulated at the transcriptional level, while Hid can be inactivated by phosphorylation that is mediated

by Ras/MAPK signaling (Bergmann et al., 1998; Kurada and White, 1998). These proteins can directly bind and inhibit the Drosophila caspase inhibitor DIAP1 (Wang et al., 1999). Several lines of evidence indicate that the mechanism by which these proteins inhibit DIAP1 include the down-regulation of the DIAP1 protein through the ubiquitin–proteasome pathway and the translational inhibition of DIAP1 (Hays et al., 2002; Holley et al., 2002; Kuranaga et al., 2002; Ryoo et al., 2002; Wilson et al., 2002; Wing et al., 2002a; Yoo et al., 2002). Mammalian mitochondrial IAP inhibitors, such as Smac/DIABLO and HtrA2/Omi, are thought to be functional orthologs of Reaper, Hid, and Grim. Sickle, another Drosophila killer protein, shares N-terminal residues in common with other killer proteins in flies (Reaper, Hid and Grim) and mammals (Smac/DIABLO and HtrA2/Omi) (Christich et al., 2002; Srinivasula et al., 2002; Wing et al., 2002b). Sickle can induce cell death by antagonizing DIAP1 function through its N-terminus. In addition to the intrinsic cell-death system, Drosophila has an extrinsic pathway that is mediated by the tumor necrosis factor (TNF) ligand and its receptor; Eiger is a Drosophila TNF homologue that triggers the JNK pathway (Igaki et al., 2002; Moreno et al., 2002; Kauppila et al., 2003) and Wengen is a TNF receptor homologue mediating Eiger signaling (Kanda et al., 2002; Kauppila et al., 2003). Thus, Drosophila possesses intrinsic and extrinsic cell death systems that are closely related to the systems seen in mammals. Hence, genetic studies of cell-death molecules in flies should be very helpful for dissecting their evolutionarily conserved functions, as well as to understand their ancestral roles.

The relative expression (or activity) of various antiapoptotic and pro-apoptotic Bcl-2 family proteins is a critical determinant of apoptosis sensitivity. Precisely how the functions of the separate subfamilies and individual Bcl-2 proteins

are coordinated to control apoptosis is far from clear, but one key target appears to be the release of cytochrome c from mitochondria and; thereby, downstream caspase activation. Bcl-2 prevents mitochondrial cytochrome c release; whereas, the addition of Bax or BH3 peptides to isolated mitochondria is sufficient to promote cytochrome c release. Bcl-2 family proteins act in a coordinated manner to control apoptosis, and various homotypic and heterotypic interactions, mediated via the BH domains of these proteins, have been described. This is considered key for their biological function, as mutation of BH domains prevents dimerization and inactivates Bcl-2 family proteins. The basic mode of interaction of Bcl-2 family proteins is the insertion of an alpha-helical BH3 domain into a shallow binding groove formed by the BH1, 2 and 3 domains of a second Bcl-2 family protein (Figure 9) (Sattler et al., 1997). Through the control of cytochrome release, Bcl-2 proteins modulate the "intrinsic" cell-death pathway; but direct regulation of caspases, for example, by physical sequestration, may also play a role (Marsden et al., 2002). Under some conditions, death receptors which can activate the "extrinsic" pathway can also cross-talk to the "intrinsic" pathway, by caspase-mediated cleavage of a BH3-only Bcl-2 family molecule, Bid, which targets the mitochondria (Figure 10). Therefore, in some cell settings, Bcl-2 proteins can also control susceptibility to death receptor induced apoptosis (Packham and Stevenson, 2005).

2.2.1.1 Mcl-1

Mcl-1 (myeloid cell leukemia-1) is an anti-apoptotic member of the Bcl-2 family protein, originally identified in 1993 in differentiating myeloid cells. Kozopas et al. (1993) isolated Mcl-1 gene from the ML-1 human myeloid leukemia cell line.

Expression of Mcl-1 increased early in the induction, or programming, of differentiation in ML-1 (at 1-3 h), before the appearance of differentiation markers and mature morphology (at 1-3 days). Mcl-1 showed sequence similarity, particularly in the carboxyl portion, to Bcl-2, a gene involved in normal lymphoid development and in lymphomas with the t(14;18) chromosome translocation. Further, in contrast to proliferation-associated oncogenes, the expression of Mcl-1 and Bcl-2 relates to the programming of differentiation/development and cell viability/death.

2.2.1.1.1 Structure

The human Mcl-1 gene is located on chromosome 1q21 (Figure 11). The prototypical Mcl-1 protein (sometimes referred to as Mcl-1L) comprises 350 amino-acid residues and contains regions of similarity to other Bcl-2 family proteins (Kozopas et al., 1993). BH domains are short motifs which mediate protein:protein interactions between family proteins and are important for apoptosis regulation (Gross et al., 1999). Mcl-1 contains BH domains 1–3, but appears to lack the N-terminal BH4 domain present in Bcl-2 and Bcl-XL. Like many other Bcl-2 family proteins, Mcl-1 also contains a C-terminal transmembrane (TM) domain that serves to localize Mcl-1 to various intracellular membranes, most notably the outer mitochondrial membrane (Yang et al., 1995). This localization is consistent with a role for Mcl-1 in controlling key mitochondrial events during apoptosis, although localization of Mcl-1 to other intracellular membranes has also been observed. The N-terminal parts of Mcl-1 contain two PEST domains, rich in proline, glutamic acid, serine and threonine amino-acid residues. PEST domains are often found in rapidly turned over proteins and Mcl-1 has a short half-life in cells (typically in the range of one to a few hours) (Craig, 2002;

Cuconati et al., 2003; Nijhawan et al., 2003). Degradation via the proteasome appears to be the major route responsible for the rapid turnover of Mcl-1. Alternate splicing via skipping of the second Mcl-1 exon gives rise to a second protein isoform, Mcl-1S/_TM (Figure 11). The N-terminal parts of this 271 amino-acid residue protein (including the PEST and BH3 domains) are identical to Mcl-1L, but Mcl-1S/_TM lacks the BH1, 2 and transmembrane domains (Bae et al., 2000; Bingle et al., 2000). Although the significance of this isoform remains to be determined, the structure of Mcl-1S/_TM resembles certain pro-apoptotic "BH3 only" proteins (Gross et al., 1999) and, in marked contrast to Mcl-1L, overexpression of Mcl-1S/_TM promotes cell death. Other isoforms of Mcl-1 can be detected by immunoblotting and these may arise from phosphorylation, alternate translation initiation and/or caspase cleavage (Michels et al., 2005).

Bae et al. (2000) identified a short splicing variant of Mcl-1, which they termed Mcl-1S. Sequence analysis indicated that the 271 amino acid variant lack BH1 and BH2 and the transmembrane domain due to the splicing out of exon 2 during mRNA processing. Unlike the full length 350 amino acid Mcl-1 protein (Mcl-1L), yeast two hybrid analysis showed that Mcl-1S does not interact with proapoptotic Bcl-2 family proteins but dimerizes with the antiapoptotic Mcl-1L. Overexpression of Mcl-1S induced apoptosis in transfected CHO cells that could be antagonized by a caspase inhibitor or specifically by Mcl-1. Therefore, the authors concluded that the fate of Mcl-1 expressing cells may be regulated through alternative splicing mechanisms and the interactions of the resulting gene products.

2.2.1.1.2 Biological functions

The rapid induction and degradation of Mcl-1 suggests it plays an important role in apoptotic control in multiple cell types in response to rapidly changing environmental cues (Craig, 2002). Consistent with this Mcl-1 is essential for embryogenesis (Rinkenberger et al., 2000) and for development and maintenance of both B and T lymphocytes in animals (Opferman et al., 2003). Mcl-1 also plays a critical role in the survival of malignant cells since depletion of Mcl-1 via antisense oligodeoxy nucleotides triggers apoptosis in cancer cells (Derenne et al., 2002).

The exact molecular mechanism by which Mcl-1 promotes cell survival is not completely understood, but is thought to involve suppression of cytochrome c release from mitochondria, possibly via heterodimerization with and neutralization of pro-apoptotic Bcl-2 family proteins, for example, Bim or Bak (Cuconati et al., 2003; Opferman et al., 2003) (Figure 12). Interestingly, Mcl-1 may be an apical player in apoptosis control, modulating early events in a cascade leading to cytochrome c release. Therefore, at least in some systems, rapid downregulation of Mcl-1 expression may be required for initiation of the apoptosis cascade (Nijhawan et al., 2003). In addition to its survival promoting functions, Mcl-1 may also play a positive role in apoptosis. The cell death promoting protein that results from caspase cleavage of Mcl-1 may participate in a positive feedback loop leading to further caspase activation (Michels et al., 2005). It is possible that Mcl-1 has additional functions, which allow it to impinge directly on cell differentiation and cell cycle control. For example, Mcl-1 binds proliferating cell nuclear antigen (PCNA) causing cell cycle arrest while the transcription factor E2F1, a key cell cycle regulator, represses Mcl-1 expression (Fujise et al., 2000; Croxton et al., 2002).

The p53 protein is an important proapoptotic regulator. It was found after cell stress, p53 interacted with BAK. This interaction caused oligomerization of BAK and release of cytochrome c from mitochondria. Mcl-1 has been showed to bind to Bak and Bax in yeast two hybrid analyses, but not to Bcl-x_L or Bcl-2. Leu et al. (2004) reported that the formation of the p53-BAK complex coincided with loss of an interaction between BAK and the antiapoptotic protein Mcl-1. Therefore, the authors suggested that p53 and Mcl-1 have opposing effects on mitochondrial apoptosis by modulating BAK activity. Cuconati et al. (2003) has demonstrated that Bak is, in fact, complexed with Mcl-1 in healthy cells.

Using the methods of somatic cell hybrid analysis and fluorescence in situ hybridization, Craig et al. (1994) mapped Mcl-1 to 1q21. In the mouse, Mcl-1related sequences were mapped to positions on 2 mouse chromosomes, 3 and 5, using haplotype analysis of an interspecific cross. The locus on mouse chromosome 3, Mcl-1, was homologous to Mcl-1 on human chromosome 1; the second locus, Mcl-rs, on mouse chromosome 5, may represent a pseudogene. The proximal long arm of human chromosome 1, where Mcl-1 is located, is duplicated and/or rearranged in a variety of preneoplastic and neoplastic diseases, including hematologic and solid tumor. Thus, Mcl-1 is a candidate gene for involvement in cancer.

Rinkenberger et al. (2000) disrupted the Mcl-1 locus in murine ES cells to determine the developmental roles of this Bcl-2 family member. Deletion of Mcl-1 resulted in periimplantation embryonic lethality. Homozygous Mcl-1 deficient embryos did not implant in utero, but could be recovered at E3.5 to E4.0. Null blastocysts failed to hatch or attach in vitro, indicating a trophectoderm defect, although the inner cell mass could grow in culture. Of note, homozygous Mcl-1-

deficient blastocysts showed no evidence of increased apoptosis, but exhibited a delay in maturation beyond the precompaction stage. This model indicates that Mcl-1 is essential for preimplantation development and implantation, and suggests that it has a function beyond regulating apoptosis.

Using a Cre/lox system, Opferman et al. (2003) generated mice with conditional Mcl-1 deficiency. The mice displayed a profound reduction in B and T lypmphocytes after Mcl-1 was removed. Differentiation of B lymphocytes was arrested at the pro-B-cell stage, while that of T lymphocytes stopped at the double-negative stage. Peripheral B and T lymphocytes were rapidly deleted. The authors noted that this developmental impairment is similar to defects observed in Il7- or Il7r-deficient mice and showed that Il7 induces Mcl-1 expression in Rag2-deficient thymocytes and in wild type T lymphocytes. Expression in wild type T lymphocytes was induced to a lesser extent with Il15 and still less with Il2. Adoptive transfer experiments indicated that Mcl-1 is required for the maintenance of peripheral B and T cells. Coimmunoprecipitation analysis indicated that Mcl-1 binds to the proapoptotic molecule Bim but not to Bad, both of which are BH3 only members of the Bcl-2 family.

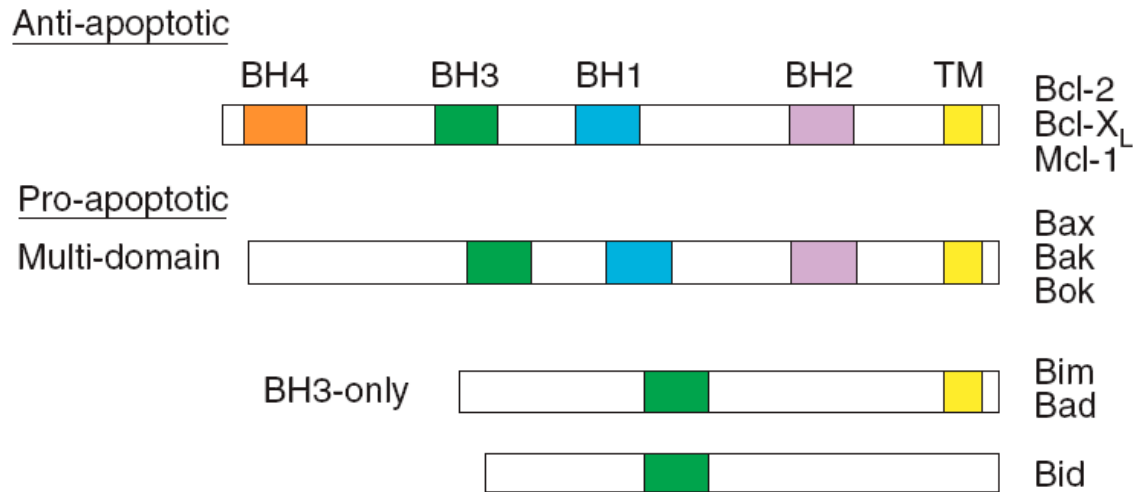**Table 2. Bcl-2 family proteins.**

Antiapoptotic

    Mammalian   Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, BAG1, Bfl-1, Boo

    Viral   E1B-19kD, BHRF-1, KSBCL-2

    C. elegans   CED-9

Proapoptotic

    Mammalian   Bax, Bak, Bik, Bok, Bad, Harakiri, Bcl-x_s

    C. elegans   Egl-1

    Drosophila   dBorg-1

Potten and Wilson (2004)

Figure 7. Schematic diagram of representative Bcl-2 family proteins.

Examples of anti-apoptotic (Bcl-2, Bcl-XL and Mcl-1), pro-apoptotic multidomain (Bax, Bak and Bok) and pro-apoptotic BH3-only (Bid, Bim, Bad) proteins are shown. Bcl-2 homology (BH) and transmembrane (TM) domains are indicated. Note that Mcl-1 may not contain a classical BH4 domain. (Packham and Stevenso, 2005)
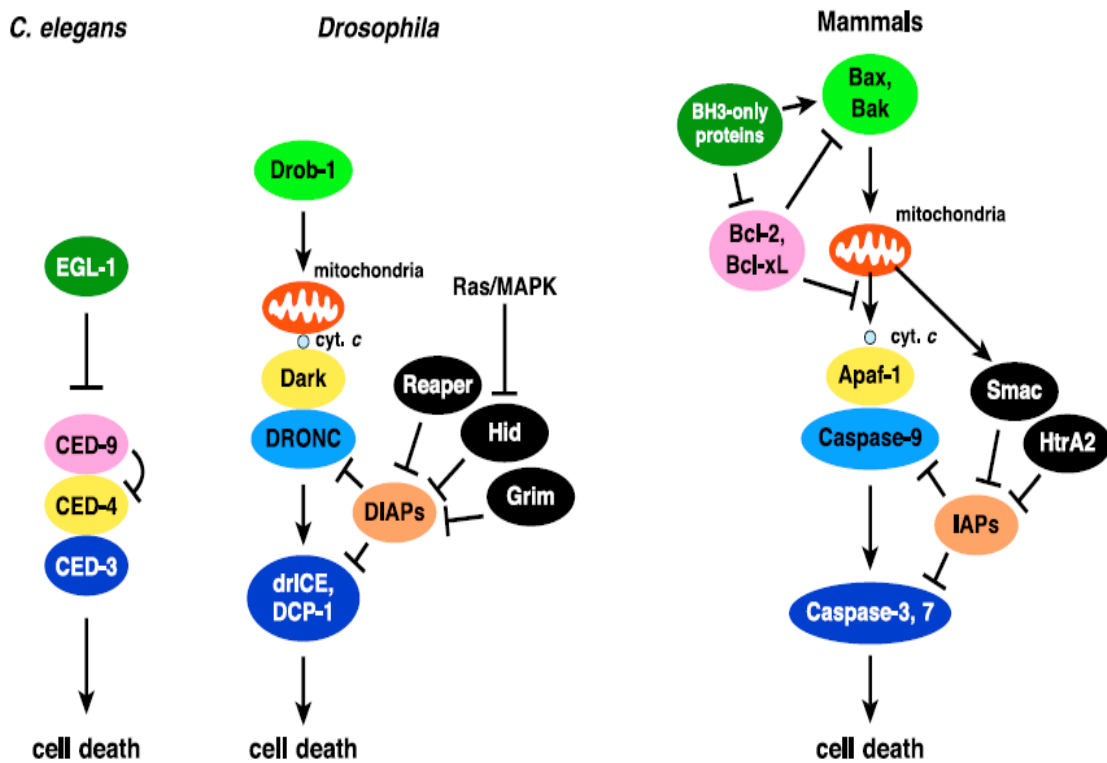
**Figure 8. Models for the intrinsic cell death pathways in *C. elegans*, *Drosophila*, and mammals.**

The evolution of the Bcl-2 family proteins may be closely linked with the role of mitochondria in the cell death pathway. (Igaki and Miura, 2004)
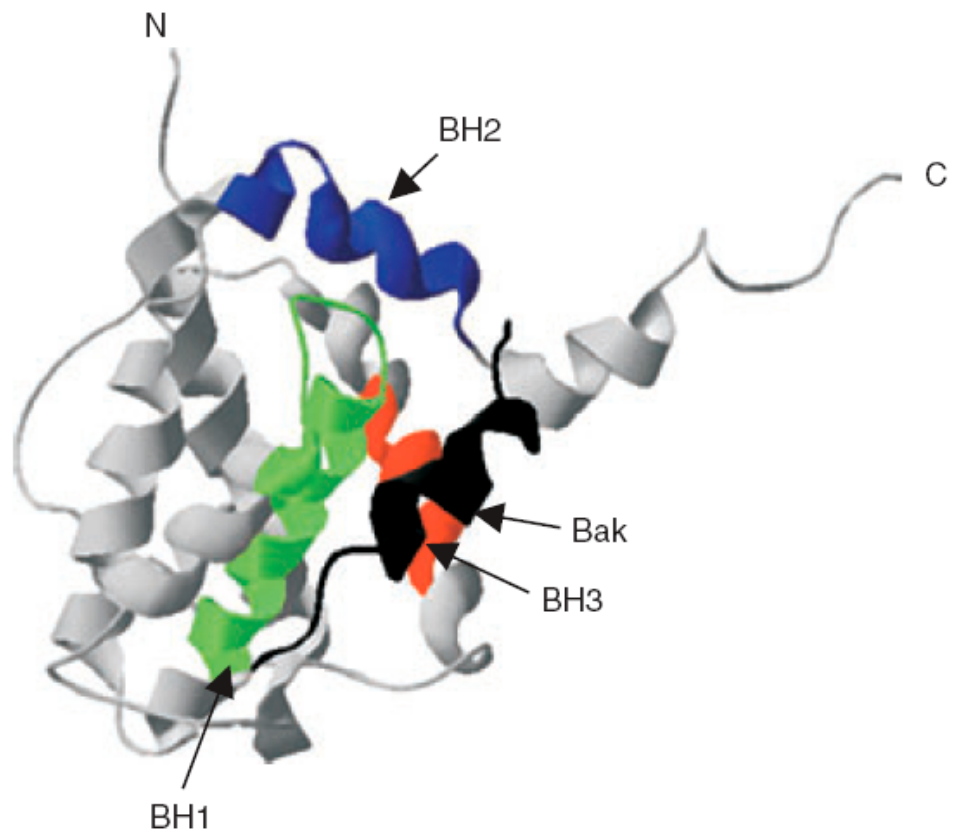
**Figure 9. Three-dimensional structure of Bcl-2 family proteins.**

Bcl-XL structure showing insertion of the Bak BH3 alpha-helix (shown in black) into a binding groove formed by domains BH1-3 of Bcl-XL (Sattler et al., 1997). N, amino-terminus; C, carboxy-terminus. (Packham and Stevenson, 2005)
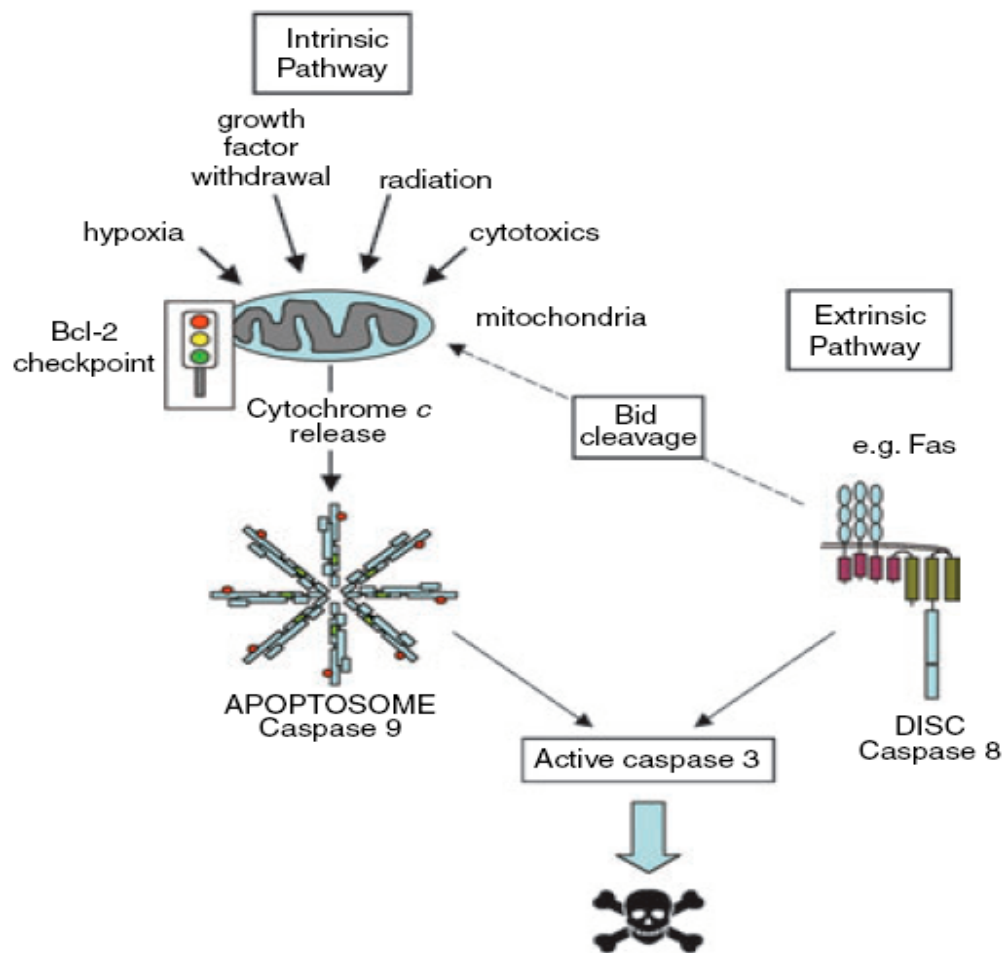
Figure 10. Pathways of apoptosis.

The 'intrinsic' and 'extrinsic' apoptosis pathways converge on the effector caspases, such as caspase 3, which promote the biochemical and morphological characteristics of apoptosis. The 'extrinsic' pathway is activated by cell-surface death receptors, such as Fas, and is mediated by activation of the initiator caspase, caspase 8, within a death-inducing signalling complex (DISC). The "intrinsic" pathway is activated by a wide range of stimuli which trigger the release of cytochrome c from mitochondria, leading to the activation of a distinct initiator caspase, caspase 9, within the apoptosome. In some situations, the activation of caspase 8 can lead to the cleavage of Bid, which is also able to promote cytochrome c release. Bcl-2 family

proteins play a key role in controlling mitochondrial function associated with the "intrinsic" cell-death pathway, either preventing or promoting the release of cytochrome c. (Packham and Stevenson, 2005)
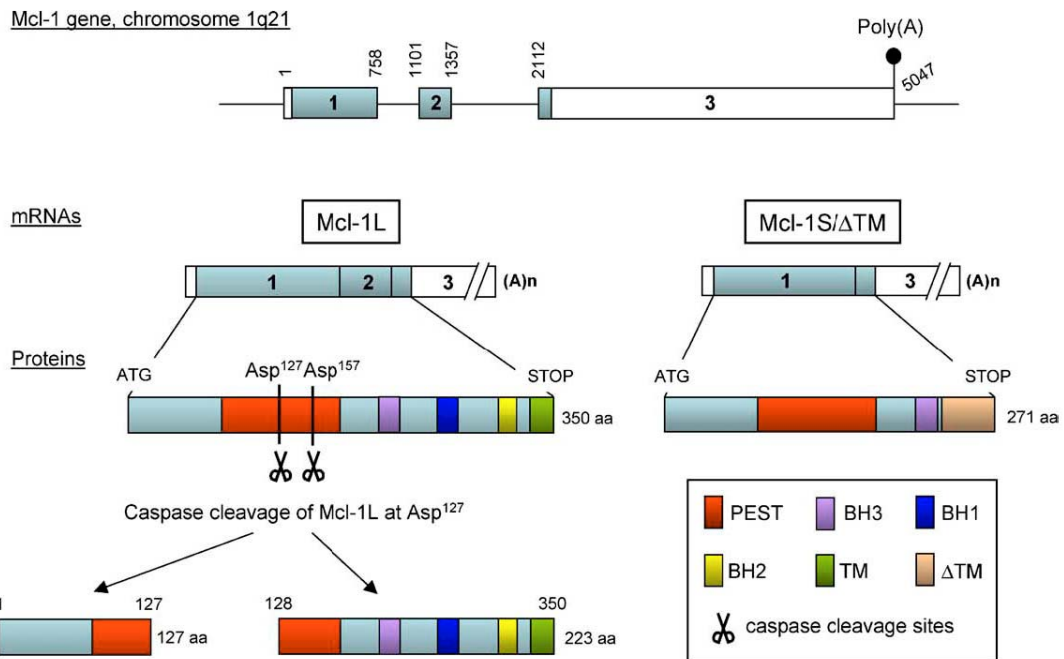
Figure 11. Molecular organization of Mcl-1.

The human Mcl-1 gene (top) is located on chromosome 1q21 and comprises three exons all of which contribute protein-coding information (highlighted). There are at least two polyadenylation sites for Mcl-1 mRNAs; the 3_ site is shown. The nucleotide co-ordinates of the exon boundaries and 3_ polyadenylation site (from GenBank accession AF198614) are given relative to transcriptional start site at position 1 (Bingle et al., 2000). Alternate splicing gives rise to distinct Mcl-1 mRNAs (middle) either containing or lacking exon 2 and encoding the Mcl-1L and Mcl-1S/_TM isoforms, respectively (Bae et al., 2000; Bingle et al., 2000). The structure and size (in amino-acid residues) of the Mcl-1L and S/_TM isoforms are shown (bottom). The PEST, BH (Bcl-2 homology) and TM (transmembrane) domains are indicated, along with the caspase cleavage sites at Asp127 and Asp157. These residues are also present in Mcl-1S/_TM but it is not known whether this isoform is similarly

cleaved by caspases. The exon 1/exon 3 splicing giving rise to Mcl-1S/_TM causes exon 3 sequences to be translated in a different reading frame and do not encode a TM domain, so sequences at the C-terminus of this isoform are unique to this isoform (shown as _TM). Finally, the Mcl-1 cleavage products generated by caspases mediated cleavage within the PEST domain at Asp127 are shown (Michels et al., 2005).
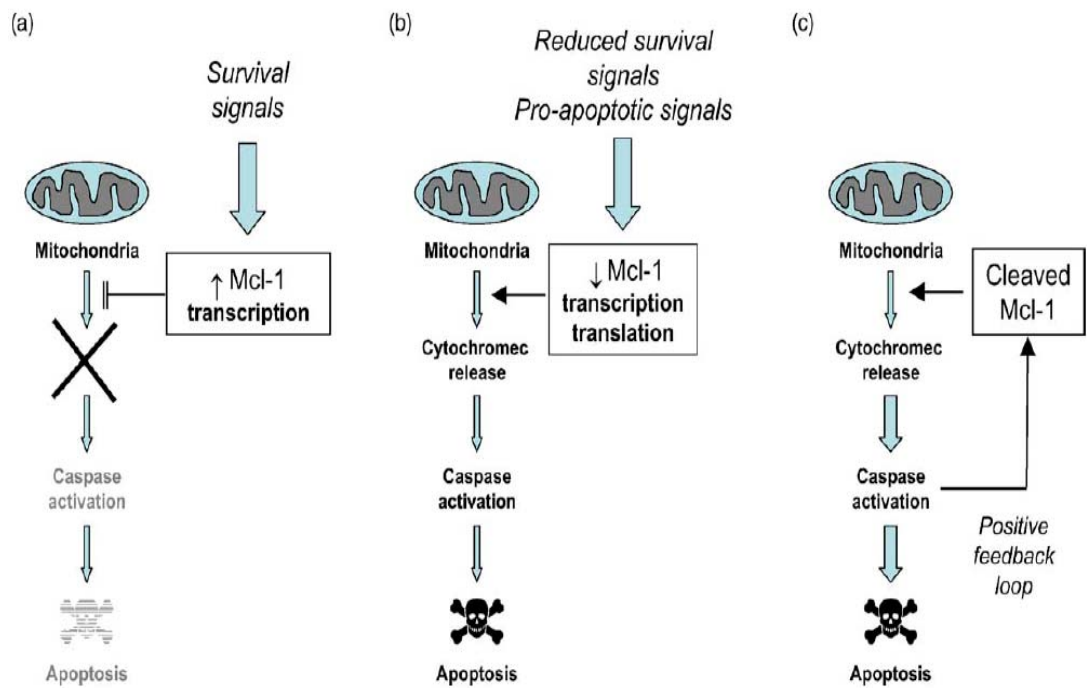
Figure 12. Regulation of apoptosis by Mcl-1.

(A) Induction of Mcl-1 expression by survival signals may contribute to resistance to apoptosis. (B) Rapid downregulation of Mcl-1 expression following removal of survival factors or exposure to other pro-apoptotic signals may contribute to apoptosis by promoting cytochrome c release. (C) Caspases activated during apoptosis can cleave remaining Mcl-1, generating a potent cell death promoting protein. (Michels et al., 2005)

2.2.2 Caspase

Caspases (cysteine aspartases) are specialized proteases that have cysteine at their active site and cleave on the C-terminal side of aspartate residues. These enzymes are synthesized initially as inactive pro-enzymes, but are converted to the active protease when cells are selected to die (Steller, 1995). Caspases inactivate cellular survival pathways, and specifically activate other factors whose function is to promote cell death (Pollard and Earnshaw, 2002).

Caspases work coordinately and sequentially in a process leading to cell apoptosis (Nunez et al., 1998). Initiator caspases (e.g., caspases-1, -8, -9, -10) are the upstream activators of the effector caspases (e.g., caspases-2, -3, -6, -7) (Kaplowitz, 2000). Effector caspases are the apoptosis executioners and their activities (Seol et al., 2001) lead to the characteristic apoptotic morphological changes such as membrane blebbing, cytoplasmic and nuclear condensation, DNA fragmentation, and formation of apoptotic bodies (Hui et al., 2004).

2.2.2.1 Initiator and effector caspases

Although the first mammalian caspase (caspase-1 or ICE) was identified as an important regulator of the inflammatory response (Cerreti et al., 1992; Thornberry et al., 1992) at least 7 of the 14 known mammalian caspases have important roles in apoptosis (Earnshaw et al., 1999; Shi, 2002). The apoptotic caspases are generally divided into two classes: the initiator caspases, which include caspase-2, -8, -9, and -10 in mammals and Dronc and Dredd in fruitflies; and the effector caspases, which include caspases-3, -6 and -7 in mammals and Drice, Decay, Damm, Dcp1 and Strica in fruit flies (Figure 13). CED-3 is the only apoptotic caspase in nematodes and

functions as both an initiator and an effector caspase. An initiator caspase is characterized by an extended N-terminal region, which comprises one or more adaptor domains that are important for its function, whereas an effector caspase usually contains 20–30 residues in its PRODOMAIN sequence. All caspases are produced in cells as catalytically inactive ZYMOGENS and must undergo proteolytic activation during apoptosis. The activation of an effector caspase, such as caspase-3 and Drice, is carried out by an initiator caspase such as caspase-9 and Dronc, respectively (Figure 14) through cleavage at specific internal Asp residues that separate the large (~p20) and small (~p10) subunits. The p20 and p10 subunits closely associate with each other to form a caspase monomer. By contrast, the initiator caspases are auto-activated. As the activation of an initiator caspase in cells inevitably triggers a cascade of downstream caspase activation, it is tightly regulated and often requires the assembly of a multi-component complex under apoptotic conditions (Adams and Cory, 2002). For example, the activation of procaspase-9 is facilitated by the APOPTOSOME, a ~1.4 MDa complex that includes Apaf-1 (apoptotic-protease-activating factor-1) and cytochrome *c* (Adams and Cory, 2002; Shi, 2002). Once activated, the effector caspases are responsible for the proteolytic cleavage of a broad spectrum of cellular targets, which ultimately leads to cell death.

All caspases are synthesized as inactive pro-enzymes and each pro-caspase contain a NH2-terminal peptide terminus of a variable length (pro-domains) (Figure 15). In most cases, there are over 100 amino acids in a pro-caspase, while generally less than 30 amino acids are present in the biologically active caspases. Such long pro-domains contain distinct motifs with important regulatory functions in the events that lead an individual caspase to function as part of a complex network of enzymes that,

when sequentially activated leads to cell death. These regions cleaved from the mature enzyme include the following domains: death effector domain (DED), caspase recruitment domain (CARD), and the death-inducing domain (DID). These amino acid sequences, similar to the so called DD domain (death domain), which are characteristically present in cell death adapter proteins, consist of six alpha-helices and have a similar overall folding structure. These domains mediate the homophilic interaction between pro-caspases and their adapters and play an important role in pro-caspase activation. While charge-charge interactions govern CARD–CARD and DD-DD association, hydrophobic interactions govern DED–DED interaction. The short pro-domains of executioner caspases seem to inhibit caspase activation, perhaps operating as a self-limiting mechanism to slow down or diminish cell apoptosis (Budihardjo et al., 1999; Martin, 2001; Weber and Vincenz, 2001; Gozani et al., 2002). All caspases contain a conserved QACXG pentapeptide active-site motif (where X is either R, Q, or G). During the process that leads to the production of mature caspases, the pro-domain and the linker peptide are cleaved at specific Asp residues (Table 3) (Cohen, 1997). The subunit, tissue distribution, substrates and function of caspase are summarized in Table 4.

Table 3. Alternative names and active-sites of human caspases.

Caspase	Alternative name	Active sequence	Cleavage site
Caspase-1	ICE	QACRG	Asp-103, Asp119, Asp-297, Asp-316
Caspase-2	Nedd2, ICH-1	QACRG	Asp-152 (exact site not known), Asp-316, Asp-330 (cleavage sites are based on equivalent sites in caspase-2)
Caspase-3	CPP32, Yama, apopain	QACRG	Asp-9/Asp-28, Asp-175
Caspase-4	ICErelII, TX, ICH-2	QACRG	Asp-104, Asp-270, Asp-289
Caspase-5	ICErelIII, TY	QACRG	Asp-121 (exact site not known), Asp-311, Asp-330
Caspase-6	Mch2	QACRG	Asp-23, Asp-179, Asp-193
Caspase-7	Mch3, ICE-LAP3, CMH-1	QACRG	Asp-23, Asp-198
Caspase-8	MACH, FLICE, Mch5	QACQG	Asp-210, Asp-216, Asp-374, Asp-384
Caspase-9	ICE-LAP6, Mch6	QACGG	Asp-130 (exact site not known), Asp-315 (cleavage by granzyme B)/Asp-330 (cleavage by caspase-3)
Caspase-10	Mch4	QACQG	Asp-219, Asp-372

Hui et al. (2004)

Table 4. Substrates and function of caspases.

Caspase	Size and subunits	Tissue distribution	Substrates	Function (and notes)
Caspase-1	Pro-enzyme: 45,158 Da; active: p20 and p10	Spleen, lung, liver, heart, skeletal muscle, kidney, and testis	Preinterleukin-1ß; interleukin-18, lamins	Processing of interleukins (inflammation); can also induce apoptosis depending on isoform and if overexpressed
Caspase-2	Pro-enzyme: 48,855 Da active: p12/14 and p19	Central nervous system, liver, kidney, and lung during embryonic development	Golgin-160, lamins	Apoptosis
Caspase-3	Pro-enzyme: 31,594 Da; active: p17 and p12	Widely distributed, with high expression in cell lines of lymphocytic origin	PARP, SREBs, gelsolin, caspases-6, -7, -9, DNA-PK, MDM2, Gas2, fodrin, b-catenin, lamins, NuMA, HnRNP proteins, topoisomerase I, FAK, calpastatin, p21Waf1, presenelin2, ICAD	Apoptosis

Table 4. (Continued)

Caspase	Size and subunits	Tissue distribution	Substrates	Function (and notes)
Caspase-4	Pro-enzyme: 43,262 Da; active: p20 and p10	Lung, liver, ovary, and placenta, barely detected in brain.	Caspase-1	Inflammation/apoptosis
Caspase-5	Pro-enzyme: 47,814 Da; active: p20 and p10	Lung, liver, and skeletal muscle	Max	Inflammation/apoptosis
Caspase-6	Pro-enzyme: 33,409 Da; active: p18 and p11	Lung, liver, and skeletal muscle	PARP, lamins, NuMA, FAK, caspase-3, keratin-18	Apoptosis
Caspase-7	Pro-enzyme: 34,276 Da; active: p20 and p11	Lung, skeletal muscle, liver, kidney, spleen, heart, and testis; no expression in the brain	PARP, Gas2, SREB1, EMAP II, FAK, calpastatin, p21^{Waf1}	Apoptosis
Caspase-8	Pro-enzyme: 55,391 Da; active: p18 and p10	Highest expression in peripheral blood	Caspases-3, -4, -6, -7, -9, -10, -13, PARP Bid	Apoptosis (death receptors)

Table 4. (Continued)

Caspase	Size and subunits	Tissue distribution	Substrates	Function (and notes)
Caspase-8		leukocytes, spleen, thymus, and liver; barely detectable in brain, testis, and skeletal muscle		
Caspase-9	Pro-enzyme: 46,322 Da; active: p35 and p10	Highly expressed in the heart, moderate expression in liver, skeletal muscle, and pancreas; low levels in all other tissues	Caspase-3, pro-caspase-9, caspase-7, PARP	Apoptosis
Caspase-10	Pro-enzyme: 58,878 Da; active: p23/17 and p12	Detectable in most tissues; lowest expression is seen in brain, kidney, prostate, testis, and colon	Caspases-3, -4, -6, -7, -8, -9	Apoptosis (death receptors)

Table 4. (Continued)

Caspase	Size and subunits	Tissue distribution	Substrates	Function (and notes)
Caspase-11	Pro-enzyme: 43,262 Da; active: p20 and p10	Brain microglia	Caspases-3, -1	Involved in inflammation and apoptosis
Caspase-12	Pro-enzyme: 48,325 Da; active: p38 and p28	Predominantly in the endoplasmic reticulum	Caspases-1, -4, -5, -11	Involved in mediating apoptosis following ER stress
Caspase-13	Proenzyme: 46,371 Da; active p35 and p10	Peripheral blood lymphocytes, spleen, and placenta	Granzyme B, caspase-8	Involved in inflammation
Caspase-14	Pro-enzyme: 27,679 Da; active: p18 and p11	Epidermal cells	Caspases-8, -10, granzyme B	Involved in inflammation

Hui et al. (2004)

2.2.2.2 Caspase-1, -8, -9, and -3 are situated at pivotal junctions in various apoptosis apoptotic pathways.

2.2.2.2.1 Caspase-1

Caspase-1 was the first member of the caspase family to be identified and originally labeled ICE (for IL-1ß-converting enzyme (interleukin-1 ß)) as a novel type of cysteine protease responsible for the conversion of precursor IL-1ß to its mature form in monocytes. The mature form of IL-1ß, cleaved at Asp-116-Ala-117, is a key mediator of inflammation. It was discovered, based on the sequence similarity to the *C. elegans* death gene, ced-3. This initiated studies about its possible role in programmed cell death. However, ICE knockout mice develop normally with no apparent physiological or morphological aberrations. This indicates that ICE has no substantial role in apoptosis (Fantuzzi and Dinarello, 1996).

The mature form of IL-1ß -converting enzyme is derived from a precursor of 404 amino acids, and it is generated by the removal of the N-terminal 119 amino acids sequence of an intermediate fragment spanning residues 298–316. The active form; thus, comprises a p20 (residues 120-297) and a p10 (residues 317-404) subunit, both of which are essential for activity (Alheim and Bartfai, 1998). Human islets of Langerhans undergo apoptosis in response to IL-1ß and Fas ligation (Loweth et al., 1998).

2.2.2.2.2 Caspase-8

Caspase-8 consists of 479 amino acids with a molecular weight of 55 kDa. There are eight alternative products derived from the alternative splicing of this gene. Like most of the upstream proteases, Caspase-8 mediates the activation cascade of

caspases in the process of Fas mediated and TNF receptor-1 induced cell death. Caspase-8 contains two death effector domains (DED), which interact with the DED of FADD (Fas-associating intracellular protein with death domain) and result in the formation of a complex among Fas, FADD, and caspase-8. The resulting aggregate called the death-inducing signaling complex (DISC) performs caspase-8 proteolytic activation (Xerri et al., 1999; Kruidering and Evan, 2000). The active dimeric enzyme is; then, liberated from the DISC and free to activate downstream apoptotic proteases (Zhou et al., 1997; Slee et al., 1999).

Two pathways for interaction of Fas and caspase-8 have been proposed (Juo et al., 1998). The first pathway involves the accessory protein FADD and the proenzymatic form of caspase-8. Upon oligomerization of the receptor molecules, the FADD molecule binds to Fas by homotypic interactions between its death domains. In the second pathway, the pro-enzymatic form of caspase-8 binds to the Fas/FADD complex by interaction between the DEDs of the FADD and the caspase-8 molecules. This results in the activation of caspase-8, which then triggers the caspase cascade. This pathway can be blocked by FADD-like IL-1ß-converting enzyme (FLICE)-inhibitory protein (FLIP), but not by Bcl-2 (Hennino et al., 2000).

Active caspase-8 can induce apoptosis by activating numerous caspases, inculding caspases-3, -4, -6, -7, -9, -10, and -13. PARP and Bid are substrates of caspase-8 (Hui et al., 2004).

Cytokine-induced apoptosis of betaTc-Tet (highly differentiated insulin-secreting cell line) has been also associated with the activation of caspase-8 (Cottet et al., 2002). In rat pancreatic acinar cells, cholecystokinin (CCK) has been shown to be capable of inducing caspase activation, cytochrome-c release, and mitochondrial

depolarization. The mitochondrial dysfunction mediated by caspase-8 then leads to the activation of caspase-3 (Gukovskaya et al., 2002). Supramaximal secretory stimulation of acini with CCK leads to a rapid redistribution and activation of caspase-8, followed by degradation of the intermediate filament (IF) termed plectin that; in turn, promotes the breakdown of the cytoskeleton. Inhibition of caspase-8 before CCK hyperstimulation prevents plectin cleavage, stabilizes F-actin morphology, and reverses the inhibition of secretion (Beil et al., 2002).

2.2.2.2.3 Caspase-9

Caspase-9 (also termed APAF3, MCH6, APAF-3, and ICE-LAP6) is a member of the *C. elegans* caspase-3 (CED-3) subfamily and constitute of 416 amino acids with a molecular weight 46 kDa. Caspase-9 has multiple mRNA forms produced by alternative splicing and is ubiquitously expressed. The highest level of caspase-9 mRNA has been observed in the heart. It is moderately expressed in the liver, skeletal muscle, and pancreas; and is present in very low levels in all other tissues. Caspase-9 and Apaf1 bind to each other via their respective NH2- terminal ced-3 homologous domains in the presence of cytochrome-c and ATP (Henshall et al., 2001; Shiozaki et al., 2002) (Figure 16). Compared to caspase-3, caspase-9 possesses a longer N-terminal pro-domain with high similarity to the pro-domains of CED-3 and caspase-2, which contain caspase recruitment domain features (CARDs). The active pentapeptide domain of Caspase-9 differs from that of the other family members being QACGG instead of QACRG. The active caspases are tetramers formed by two large (usually ~20 kDa) and two small (~10 kDa) subunits. Residues from both types of subunits contribute to the formation of the substrate binding sites (two per tetramer). Small

subunits are derived from the C-terminal portion of the zymogen polypeptide, by cleavage at one site, or two closely spaced sites on the C-terminal end of the active-site cysteine. Caspase-9 is an exception when compared to other caspases in that, in vivo, the active, mature enzyme retains the N-terminal pro-domain as part of the large subunit (35 or 37 kDa) (Hui et al., 2004).

Caspase-3, pro-caspase-9, caspase-7, and PARP are all substrates of caspase-9. In the presence of dATP, caspase-9 is directly activated by Apaf-1 and cytochrome-c (Susin et al., 1997). Active caspase-9; in turn, activates caspase-3 and, by doing so, initiates the apoptotic machinery that leads to apoptosis (Fujita et al., 2001). Caspase-9 activity is regulated by protein phosphorylation (Cardone et al., 1998). The kinase Akt phosphorylates pro-caspase-9 at Ser196 and, by this, inhibits proteolytic processing of pro-caspase-9 (Hui et al., 2004).

2.2.2.2.4 Caspase-3

Caspase-3 (also termed CPP32, apopain, and Yama) is a member of the IL-1ß converting enzyme family of cysteine proteases and has been shown to have the highest sequence similarity to ced-3. The enzyme is composed of 17 and 12 kDa subunits derived from a common proenzyme termed pro-caspase-3. Caspase-3 is widely distributed in eukarioric cells, with high expression in cell lines of lymphocytic origin (Fernandes-Alnemri et al., 1994).

Caspase-3 plays a major role in Fas-mediated apoptosis of beta-cells. Yamada et al. (1999), using a beta-cell line transfected with human Fas, showed that the crosslinking of Fas by anti-Fas resulted in the elevation of caspase-3-like; but not caspase-1-like, protease activity 2-12 h after the addition of the anti-Fas. A caspase-3

inhibitor, Z-Asp-Glu-Val-Asp-fluoromethyl ketone, attenuated the Fas-mediated beta-cell apoptosis while a caspase-1 inhibitor, acetyl-Tyr-Val-Ala-Asp-chloromethylketone, failed to suppress the apoptosis. Thus, the Fas-induced death signal apparently bypassed caspase-1 in the cells. Furthermore, an anti-sense caspase-3 construct blocked caspase-3 activation and; substantially, suppressed Fas-triggered apoptosis of hFas/betaTC1 cells. These observations suggest the essential role of caspase-3 in Fas-mediated apoptosis of the beta-cell line (Hui et al., 2004).

Caspase-3 is activated during apoptotic signaling events by upstream proteases, including caspases-6, -8, and cytotoxic T-cell derived granzyme-B (Zapata et al., 1998). The crystal structure of caspase-3 shows that the active enzyme is a tetramer, containing two small and two large subunits. A small and a large subunit form a heterodimer and two of these heterodimers form the mature tetramer (or homodimer of the two heterodimers) (Hui et al., 2004).

Caspase-3 has been identified as being a key mediator of apoptosis of mammalian cells. It can cleave numerous substrates with a DXXD sequence to effect the morphological changes associated with apoptosis (Fattman et al., 2001). Caspase-3 substrates include: PARP, SREBs, gelsolin, caspases-6, -7, -9, DNA-PK, MDM2, Gas2, fodrin, b-catenin, lamins, NuMA, HnRNP proteins, topoisomerase I, FAK, calpastatin, p21^{Wafl}, resenelin2, and DFF45 (human 45 kDa DNA fragmentation factor (DFF)), etc. The significance of PARP cleavage is not clear; although, this phenomenon represents an excellent marker for caspase activation and the presumption of ongoing apoptosis (Kothakota et al., 1997).
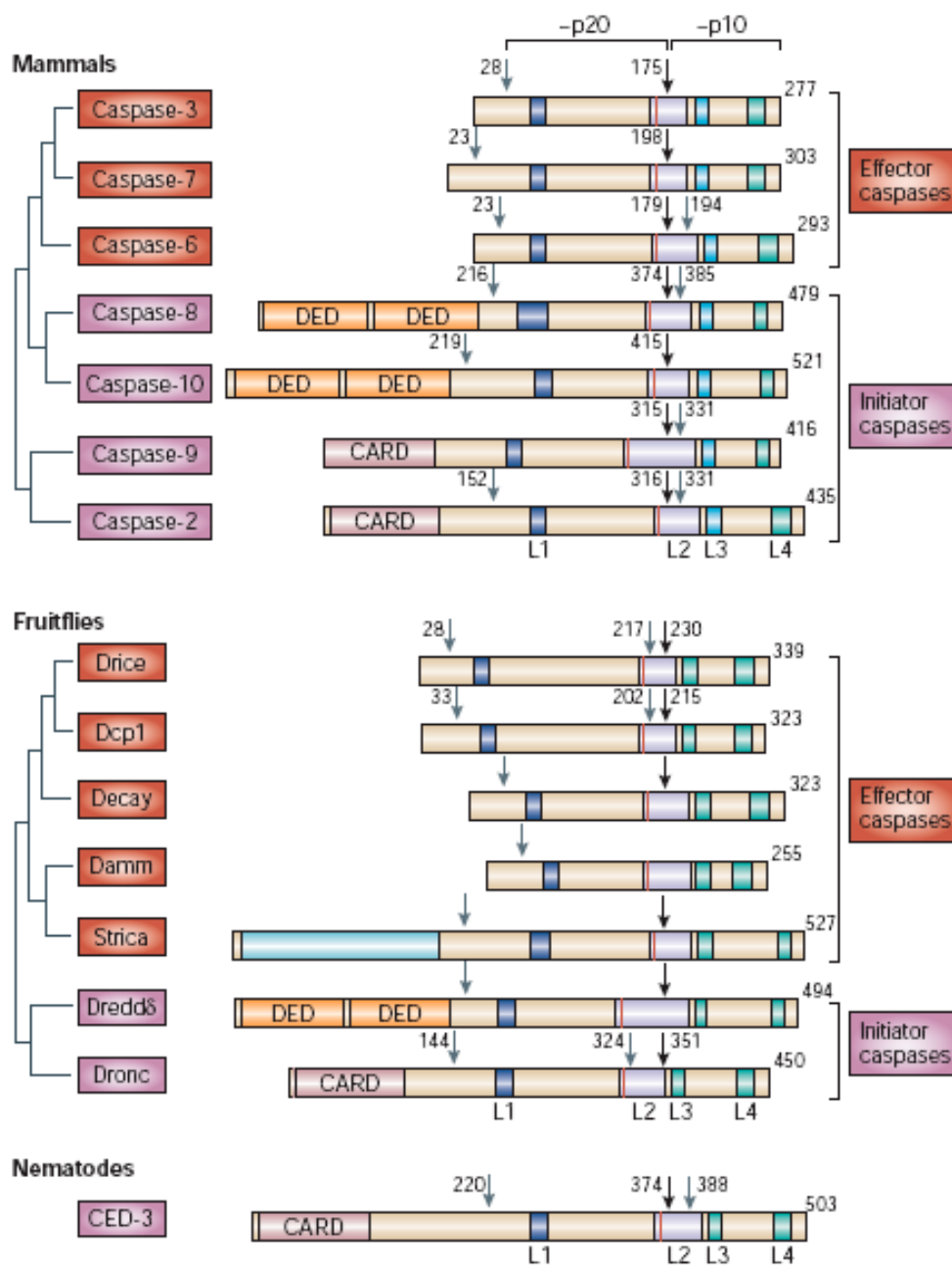
Figure 13. Apoptotic caspases in mammals, fruitflies, and nematodes.

The effector and initiator caspases are shown in red and purple, respectively. CED-3 (cell-death abnormality-3) is the only caspase in the nematode worm *C. elegans* and fulfills the role of both initiator and effector caspase. The position of the first intra-chain activation cleavage (between the large and small subunits, ~p20 and

~p10, respectively) is highlighted by a black arrow; whereas, other sites of cleavage are represented by grey arrows. These other cleavage events are thought to modulate caspase activity and the regulation of caspases by inhibitor of apoptosis (IAP) proteins and by other proteins. Unlike other protease zymogens, removal of the N-terminal prodomain of a caspase is unnecessary for its catalytic activity. The prodomains in initiator caspases invariably contain homotypic interaction motifs, such as the caspase-recruitment domain (CARD) and the death-effector domain (DED). The four surface loops (L1–L4) that shape the catalytic groove are indicated. The catalytic residue Cys is shown as a red line at the beginning of L2. The p20 and p10 subunits together form a caspase monomer. The caspases and the location of functional segments are drawn to scale. (Riedl and Shi, 2004)
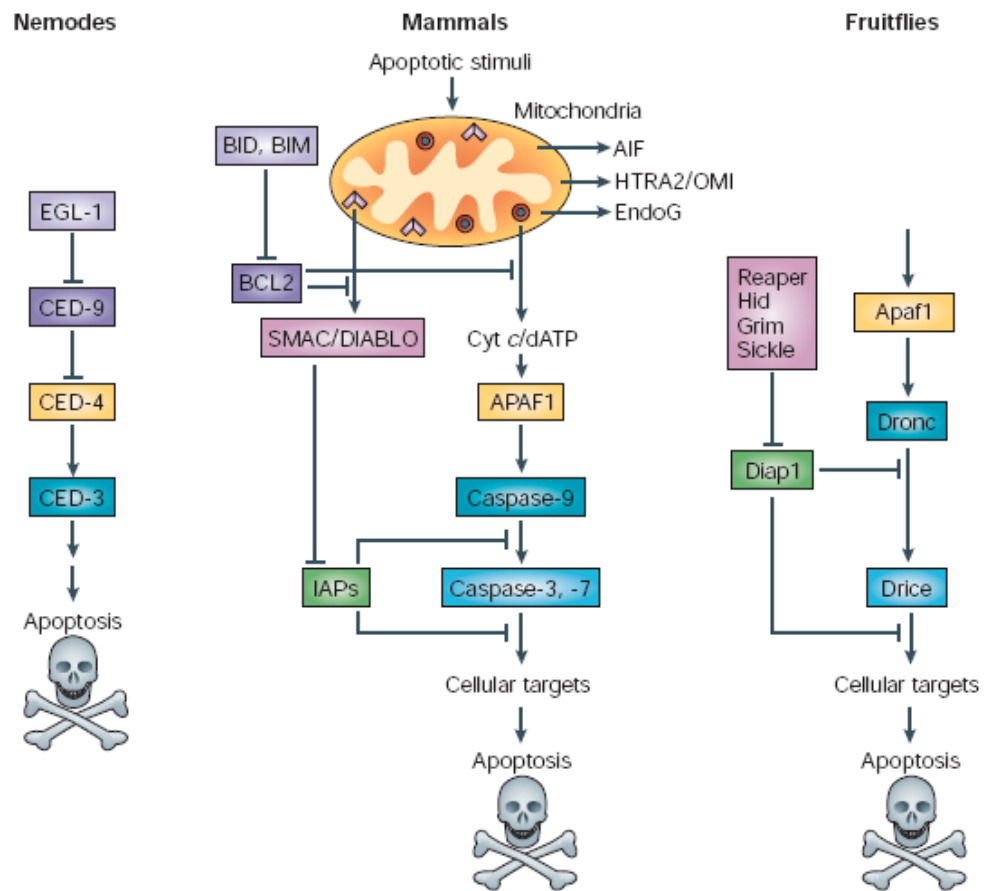
Figure 14. A conserved apoptotic pathway in nematodes, mammals, and fruit flies.

Functional homologues of caspases and caspase regulators across species are indicated by the same colour. Caspase-9 in mammals and Dronc in the fruitfly *Drosophila melanogaster* are initiator caspases; whereas, caspase-3 and -7 in mammals and Drice in fruitflies belong to the class of effector caspases. CED-3 (cell-death abnormality-3) in the nematode worm *C. elegans* functions both as an initiator and effector caspase. The inhibitor of apoptosis (IAP) proteins suppress apoptosis by negatively regulating the caspases; whereas, SMAC (second mitochondria-derived activator of caspases)/DIABLO (direct IAP-binding protein with low pI) in mammals

and the RHG proteins Reaper, Hid, Grim and Sickle in fruitflies can remove the IAP-mediated negative regulation of caspases. AIF, apoptosisinducing factor; APAF1, apoptotic-protease-activating factor-1; Cyt *c*, cytochrome *c*; EndoG, endonuclease G; HTRA2, high-temperature-requirement protein A2. (Riedl and Shi, 2004)
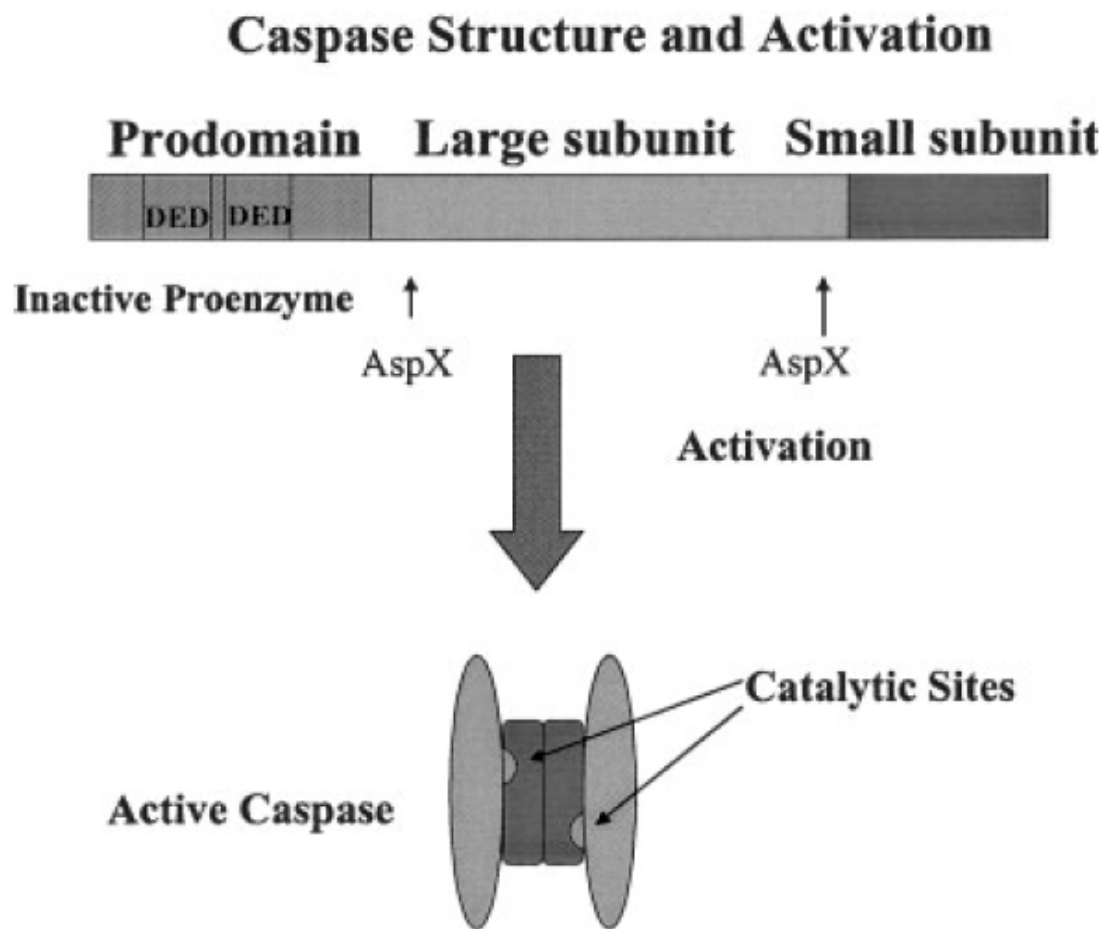
Figure 15. Caspase structure and activation.

The pro-caspase is inactive and its typical structure contains three parts: a pro-domain, a large subunit, and a small subunit. The length of pro-domain varies among caspases and some of them have death effector domain feature (DED) or caspase recriutment domain feature (CARD). Pro-enzyme is cleaved at caspase cleavage sequences (Aps-X), and two large and two small subunits combine to form the active tetrameric enzyme (Hui et al., 2004).
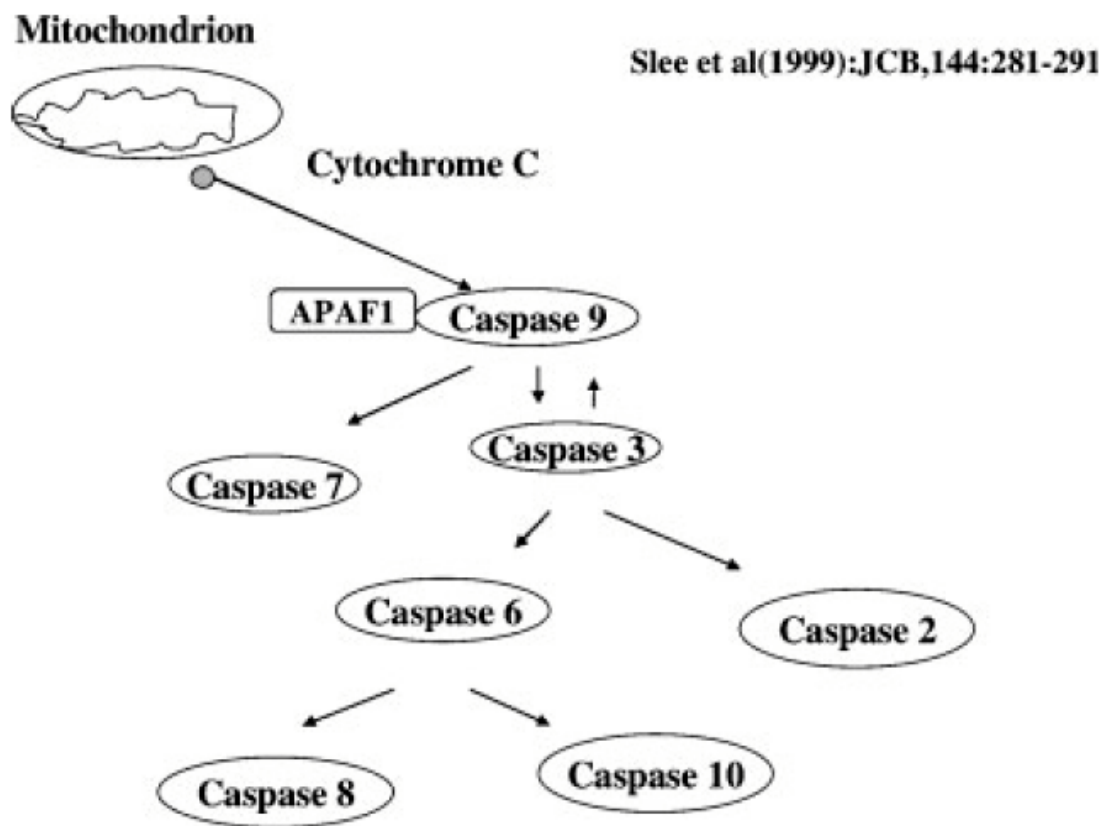
Figure 16. The activation of caspase-9.

Caspase-9 is activated in response to stimuli that triggers the release of cytochrome-c from the mitochondria. The coordinated action of caspase-9, dATP, APAF-1, and cytochrome-c then leads to the activation of other substrate proteins that are part of the caspase cascade. (Hui at al., 2004)

3. Translationally controlled tumor protein/Fortilin

The Translationally controlled tumor protein (TCTP) was first identified as a growth-related protein in mouse ascites and erythroleukemic cells (Yenofsky et al., 1983). TCTP, also know as IgE-dependent histamine releasing factor (HRF) is a 23 kDa protein (P23) in humans (Gross et al., 1989); it has a 21 kDa mouse homologue (P21) (Chitpatima et al., 1988; Gross et al., 1989) and Q23 (Thomas and Luther, 1981; Thomas, 1986). The name 'translationally controlled tumour protein' was coined (Gross et al., 1989), based on the fact that the cDNA was cloned from a human tumour and on the observation that TCTP is regulated at the translational level. Elucidation of the primary sequence did not reveal any similarity with other protein families. Only the recent determination of the solution structure of the fission yeast protein indicated similarity with a small chaperone family (Thaw et al., 2001). In addition, TCTP was shown to have anti-apoptotic activity. These findings led the authors to use other names for this protein "fortilin" (Li et al., 2001; Zhang et al., 2002a).

TCTP is ubiquitously expressed in all eukaryotic organisms and in more than 500 tissues and cell types investigated so far. However, expression levels vary widely depending on the cell/tissue type (Thiele et al., 2000) and on the developmental stage (Gnanasekar et al., 2002; Rao, 2002). TCTP is expressed in mitotically active tissues; whereas, expression levels are low in postmitotic tissue like the brain (Thiele et al., 2000). In numerous experimental settings and biological systems, it was established that TCTP levels are highly regulated in response to a wide range of extracellular signals and cellular conditions (Figure 17). Typically, growth signals (Bommer et al., 2002) and cytokines (Nielsen et al., 1998; Teshima et al, 1998) have been reported to rapidly induce TCTP synthesis. Various stress conditions, such as starvation (Bonnet

et al., 2000; Bommer et al., 2002), heat shock, heavy metals, calcium stress (Xu et al., 1999) or proapoptotic/cytotoxic signals (Oikawa et al., 2002; Sinha et al., 2000) result in either up- or down-regulation of TCTP levels (Bommer et al., 2002).

3.1 Protein structure and conservation

TCTP is a highly conserved protein that is found in normal cells including mammals, nematodes (Bini et al., 1997), trypanosomes (Haghighat and Rubeu, 1992), higher plants (Pay et al., 1992), and Saccharomyces (Sanchez et al., 1997). Sequence alignment of TCTP sequences from more than 30 different species reveals a high degree of conservation over a long period of evolution. In Figure 18A, Bommer and Thiele (2004) reported the alignment of TCTP sequences from five species representing from five kingdoms. Nine of the approximately 170 residues are completely conserved and six additional ones are only mismatched in one sequence, making up a total of nearly 9% absolutely conserved amino acids. The invariant residues are largely clustered on one side of the ß-stranded 'core' domain (Figure 18B), indicating that this side is important for molecular interactions. The fold of this domain displays significant similarity to that of the Mss4 and Dss4 proteins, two small chaperones reported to bind to the nucleotide-free form of Rab proteins (Thaw et al., 2001). TCTP is; therefore, now grouped in one protein family together with Mss4/Dss4. The other major domains, the flexible loop and the helical domain (Figure 18B), are specific for TCTP. The middle of the loop contains a highly conserved area, listed in the prosite database as TCTP signature 1. The only molecular functions of the TCTP protein mapped so far are the tubulin-binding region (Gachet et al., 1999) and the Ca^{2+}-binding area (Kim et al., 2000). Both coincide with the helical domain, which

also represents the most basic part of the molecule (Gachet et al., 1999; Thaw et al., 2001).

3.2 Gene structure and pseudogenes

The genomic structures of four mammalian *TPT1* genes are currently available, from human (NT009910), mouse (NT039606), rat (NW043879) and rabbit (Z46805). All four genes are of identical intron/exon organisation with five introns and six exons. The boundaries of exons 1–5 are indicated in Figure 18A in relation to the amino acid sequence. Exon 6 comprises the 3'-UTR of the mRNA. The chromosomal localisation has been determined for the human, rat, and mouse genes. The *TPT1*-gene contains a promoter with a canonical TATA-box and several promoter elements, which are well-conserved in mammals (Bommer and Thiele, 2004). In rabbit, the promoter contains a TATA box at -30 and potential binding sites for transcription factors such as stimulating protein 1 (Sp1), nuclear factor 1 (NF1), activator protein 1 (AP1), c-Ets1, cAMP-response element (CP2), myeloid-specific zinc finger protein 1 (MZF1) and others. In reporter gene assays, this region exhibits a strong promoter activity comparable to viral promoters (Thiele et al., 1998). Mammalian genomes contain substantial numbers of intron-less, processed TPT1 pseudogenes. The *TPT1* gene encoding the TCTP generates two mRNAs (TCTP mRNA1 and TCTP mRNA2) which differ in the length of their 3' untranslated region. The distribution of these mRNAs was investigated in 10 rabbit and 50 human tissues. Pseudogene transcription was supported further by CAT reporter gene assays showing substantial promoter activity of 5'-flanking regions of two TPT1 pseudogenes A genomic BLAST search detects 15 pseudogenes in humans, 18 in rat, and 13 in

mouse. All these represent cDNA-like, processed pseudogenes with a high degree of conservation. In rabbit, the number of TPT1 pseudogenes was estimated to be in the range of about 10–15 (Thiele et al, 2000).

3.3 Biological function

3.3.1 Molecular interactions

The first molecular function of TCTP was reported as a calcium-binding protein (Haghighat and Rubeu, 1992; Sanchez et al., 1997; Bhisutthibhan et al., 1999; Xu et al., 1999). Recently, only one publication has mapped the calcium-binding region. The results indicated that amino acid residues 81-112 of rat TCTP are the calcium-binding sites (Figure 18) (Kim et al., 2000). In mammalian cells, it was shown the part of TCTP that binds to calcium also binds to microtubules during most of the cell cycle, inclusive of the metaphase spindle, but is detached from the spindle after metaphase (Gachet et al., 1999). This finding was recently corroborated by the demonstration that TCTP is phosphorylated by the protein kinase Plk, which is likely to cause detachment of TCTP from the mitotic spindle. The results also suggest that phosphorylation decreases the microtubule-stabilizing activity of TCTP and promotes the increase in microtubule dynamics that occurs after metaphase (Yarm, 2002). Other molecular interactions of TCTP published to date include self-interaction. The domain mapping of the interaction revealed that the C-terminal region of residue 126-172 is involved in self-interaction (Yoon et al., 2000). In addition, TCTP also interacts with the MCL1 protein (Zhang et al., 2002a).

3.3.2 Cellular importance

As TCTP levels are considerably up-regulated during entry of cells into the cell cycle, the protein is believed to be important for cell growth and division. This conclusion is confirmed by the following observations:

(1) Overexpression of TCTP (or P23) in mammalian cells resulted in slow growth, in alteration of cell morphology, and a delay in cell cycle progression (Gachet et al., 1999).

(2) A genome-wide screen for phenotypes in *Caenorhabditis elegans* using RNAi established that knockdown of TCTP results in a slow-growth phenotype (Kamath et al., 2003).

(3) Overexpression of TCTP mutated in the phosphorylation sites for the mitotic kinase Plk (Figure 18) disrupts the completion of mitosis. TCTP is a key mitotic target of Plk for regulating anaphase progression (Yarm, 2002).

(4) Gene-knockout of TCTP in fission yeast resulted in cells compromised in entry into and exit from the cell cycle (Bommer and Thiele, 2004).

(5) Down-regulation of TCTP expression was found to be associated with reversion of cells from the transformed to the normal phenotype. It appears that in the process, the imbalance in gene expression produced by the cancer cell would be at least partially corrected by inhibition of *tpt1*/TCTP. Hence, tumor reversion can be defined at the molecular level, not just as the reversal of malignant transformation, but as a biological process in its own right involving a cellular reprogramming mechanism, overriding genetic changes in cancer, by triggering an alternative pathway leading to suppression of tumorigenicity (Tuynder et al., 2002).

The majority of publications describe TCTP as a cytoplasmic protein, but nuclear localisation has also been reported (Li et al., 2001). TCTP levels are regulated in response to various stress conditions and an increase in TCTP levels was reported to be associated with increased chemoresistance (Sinha et al., 2000; Walker et al., 2000). Overexpression of mammalian TCTP results in microtubule stabilisation and alteration of cell morphology (Gachet et al., 1999). Together with TCTP's similarity to chaperones (Thaw et al., 2001) and its recent characterization as an anti-apoptotic protein (Li et al., 2001), these observations suggest that TCTP generally exerts a cytoprotective function. The cellular importance of TCTP is summarized in Figure 17.

3.3.3 TCTP in parasitic organisms

TCTP has been characterized from various parasitic organisms, such as *Plasmodium* subspecies (Walker et al., 2000) and parasitic worms (*Wuchereria bancrofti* and *Brugia malayi*) (Gnanasekar et al., 2002). Walker et al., (2000) have studied artemisinin (anti-malaria drug) resistance in the murine malaria parasite *Plasmodium yoelii*. The TCTP, one of the artemisinin target proteins and increased TCTP expression correlated with increased resistance against this drug (Bhisutthibhan et al., 1998). In *Wuchereria bancrofti* and *Brugia malayi*, TCTP has been cloned and characterized. The studies showed that TCTP from both parasites are calcium-binding proteins, have histamine-releasing function in vitro, and is able to cause inflammatory infiltration of eosinophils (Gnanasekar et al., 2002). Thus, it appears that the parasitic TCTP might be involved both in certain pathological processes in the infected host, and in the development of drug resistant.
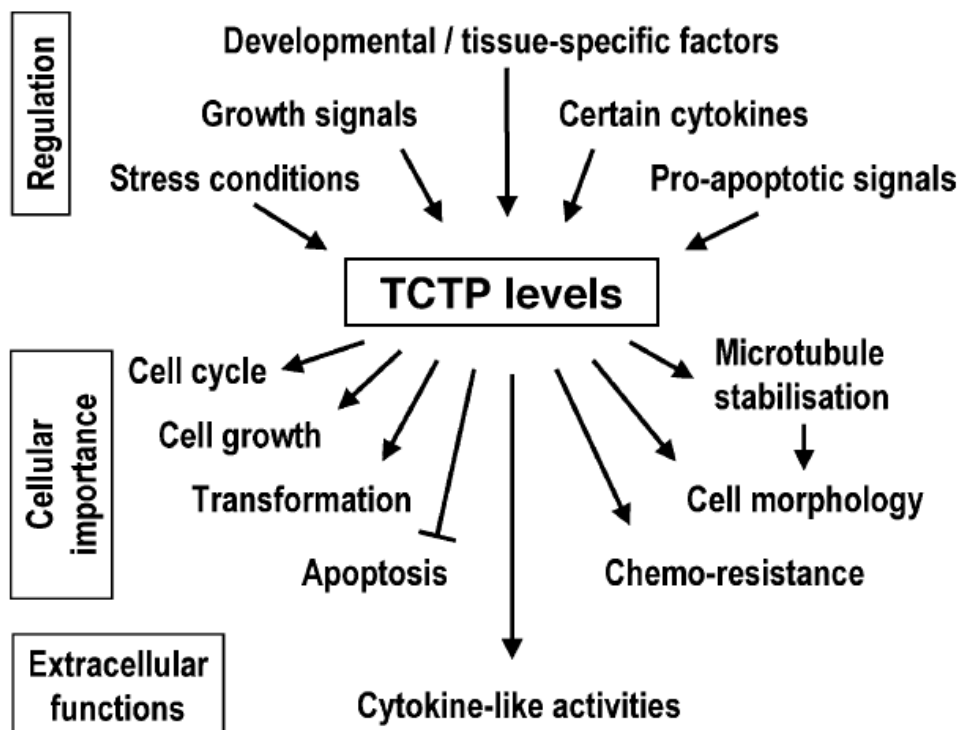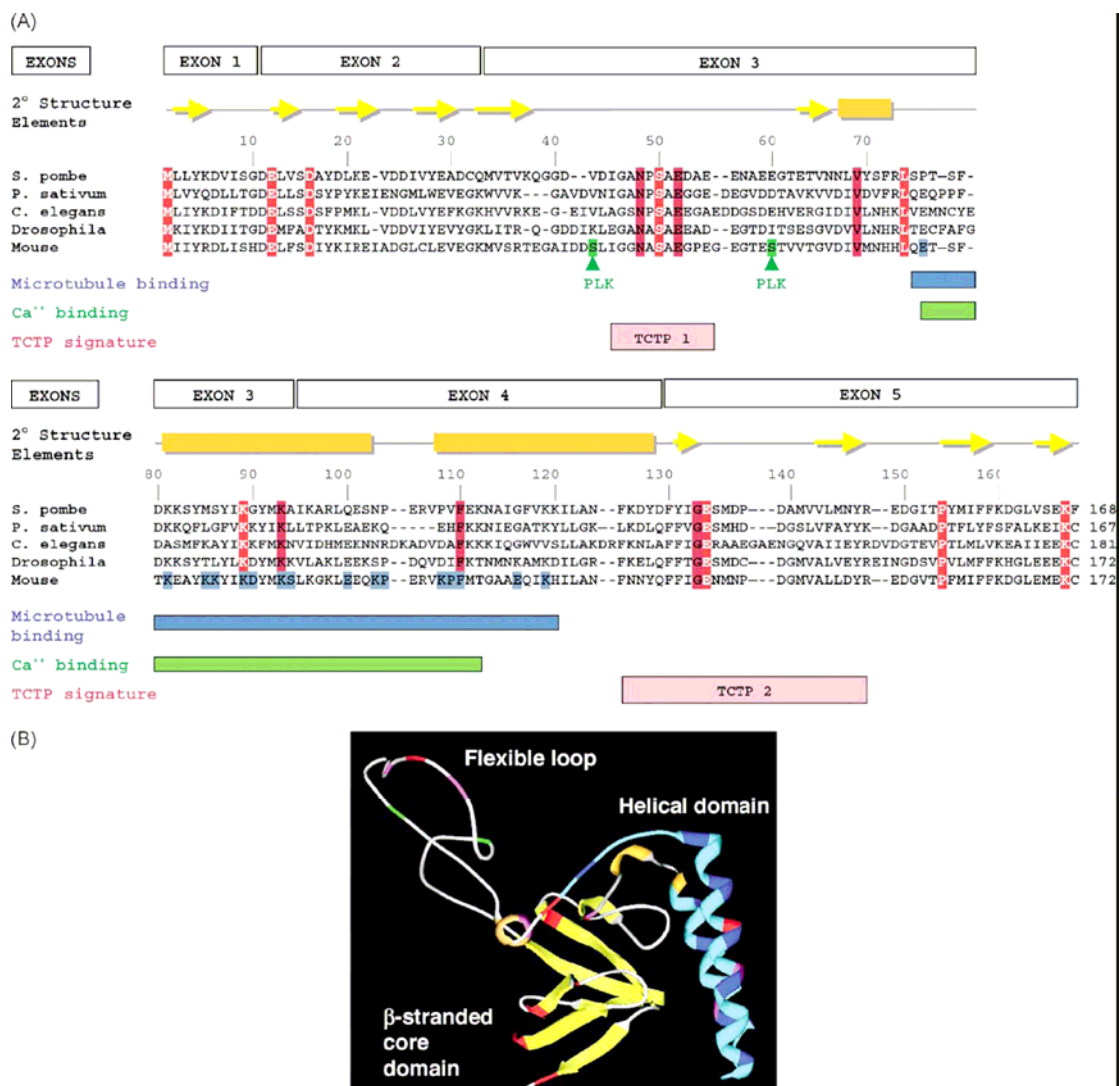
Figure 17. Regulation and functional importance of TCTP.

This figure summarises the extracellular signals and conditions that result in regulation of TCTP levels and the cellular and extracellular importance of the protein. See text for details. (Bommer and Thiele, 2004)

Figure 18. Sequence conservation, 3D structure, and functional mapping of the TCTP protein.

(A) TCTP sequences of the following species were aligned: fission yeast (*S. pombe*; Q10344), pea (*P. sativum*; P50906), a nematode (*C. elegans*; Q93573), the fruit fly (*Drosophila*; Q9VGS2), and mouse (P14701). Numbering is for the *S. pombe* sequence. Invariant residues are labelled in red, residues conserved in all but one of 30 TCTP sequences are highlighted in pink. Exon boundaries are indicated by boxes at the top. Secondary structure elements of the fission yeast protein (Thaw et al., 2001)

are represented as follows, ß-strands: yellow arrows, helices: orange bars, coiled regions: grey line. The microtubule-binding region (Gachet et al., 1999) and the Ca2+-binding region (Kim et al., 2000) are indicated by a blue bar and a green bar, respectively. Residues of the mouse sequence that match amino acids within part of the tubulin-binding region of human MAP-1B (Gachet et al., 1999) are highlighted in blue. Serine residues in the mouse sequence that are phosphorylated by the mitotic kinase Plk (Yarm, 2002) are labelled in green. Note that these serines are only conserved in mammalians. TCTP signatures listed in the prosite database are shown in red. (B) Ribbon structure of the fission yeast TCTP ((Thaw et al., 2001); PDB: 1H6Q). The major domains are indicated in the figure. The colour coding is shown in part (A). The microtubule-binding region is labelled in light blue. Dark blue regions mark those residues of the microtubule-binding region labelled blue in the mouse sequence in (A). The calcium-binding domain is not indicated; it is practically identical to the microtubule-binding region. (Bommer and Thiele, 2004)

4. Gene silencing: RNA interference

RNA interference (RNAi) is the process of sequence-specific, post-transcriptional gene silencing (PTGS) in animals and plants, and initiated by double-stranded RNA (dsRNA) that is homologous in sequence to the silenced gene (Elbashir et al., 2001a; 2001b). PTGS/RNAi involves the targeted elimination of mRNA by a homologous dsRNA that is processed into short interfering RNAs (siRNAs) approximately 21-23 nucleotides in length (~2 nucleotides of 3' overhang on each strand) by an RNase III-type enzyme termed Dicer. The siRNAs are thought to guide an RNA-Induced Silencing Complex (RISC) to the complementary mRNA, which is subsequently degraded (Figure 19) (Bernstein et al., 2001; Chicas and Macino, 2001; Cogoni, 2001; Tuschl 2001; Vance and Vaucheret, 2001; Kurreck, 2003). RISC is a multiple turnover RNA endonuclease complex and one of its components is a member of the Argonaute protein family (Hutvagner and Zamore, 2002; Martinez et al., 2002). The target mRNA is cleaved within the region recognized by the anti-sense siRNA strand and the cleavage site is located opposite the phosphodiester bond between 10th and 11th nt of the anti-sense strand counting from its 5' end. The mRNA fragments deprived of either cap structure or polyA tail, both required for mRNA stability, are subsequently degraded by cellular nucleases. Consequently, the specific depletion of mRNA from the cell leads to a reduction of the corresponding protein, resulting in a knock down phenotype (Elbashir et al., 2001a; 2001b; Martinez et al., 2002).

RNA silencing mechanisms were first recognized as antiviral mechanisms that protect organisms from RNA viruses, or which prevent the random integration of transposable elements. First discovered in plants, the phenomenon of homology-dependent gene silencing came into the scientific world through serendipity. Two

seminal studies were those of Napoli and Jorgensen (1990) and Van der Krol et al. (1990), each attempting to make purple petunias an even darker purple by introducing a transgene designed to overproduce the chalcone synthase enzyme. However, over-expression of chalcone synthase transgenes did not increase coloration. Instead, some flowers were completely colorless (white), and others showed interesting patterns such as loss of pigmentation along the veins, but full pigmentation in other areas. It was the complete loss of color in some or all flower cells that indicated a new phenomenon had been stumbled upon, for not only was the transgene silent in these cells but the endogenous copy of the chalcone synthase gene was silenced, as well. Napoli and Jorgensen (1990) thus introduced the term "co-suppression" to describe the coordinate silencing of a transgene and its endogenous homologs.

4.1 Experimental applications

4.1.1 Invertebrate

The term RNA interference (RNAi) was coined after the discovery that injection of dsRNA into the nematode *C. elegans* leads to specific silencing of genes highly homologous in sequence to the delivered dsRNA (Fire et al., 1998). RNAi was also observed subsequently in insects (Kennerdell and Carthew, 1998), and frog (Oelgeschlager et al., 2000). The natural function of RNAi and cosuppression appears to be protection of the genome against invasion by mobile genetic elements such as transposons and viruses, which produce aberrant RNA or dsRNA in the host cell when they become active (Jensen et al., 1999; Ketting et al., 1999; Ratcliff et al., 1999; Tabara et al., 1999; Malinsky et al., 2000). Specific mRNA degradation prevents transposon and virus replication; although, some viruses are able to overcome or

prevent this process by expressing proteins that suppress PTGS (Anandalakshmi et al., 2000; Lucy et al., 2000; Voinnet et al., 2000). dsRNA triggers the specific degradation of homologous RNAs only within the region of identity with the dsRNA. The dsRNA is processed to 21–23-nt RNA fragments (Zamore et al., 2000). These short fragments were also detected in extracts prepared from *Drosophila melanogaster* Schneider 2 cells that were transfected with dsRNA before cell lysis (Hammond et al., 2000) or after injection of radiolabeled dsRNA into *D. melanogaster* embryos (Yang et al., 2000) or *C. elegans* adults (Parrish et al., 2000). RNA molecules of similar size also accumulate in plant tissue that exhibits PTGS (Hamilton and Baulcombe, 1999). It has been suggested that the 21–23-nt fragments are the guide RNAs for target recognition (Hamilton and Baulcombe, 1999; Hammond et al., 2000), which is supported by the finding that the target mRNA is cleaved in 21–23-nt intervals (Zamore et al., 2000). The systems are commonly used in plants such as *Arabidopsis* (Dalmay et al., 2000; Dalmay et al., 2001; Park et al., 2002) and invertebrates such as *Caenrhabditis* (Kamath et al., 2001; Timmons et al., 2001; Alder et al., 2003; Lee et al., 2003), *Drosophila* (Dzitoyeva et al., 2001; Kalidas and Smith, 2002; Wei et al., 2003).
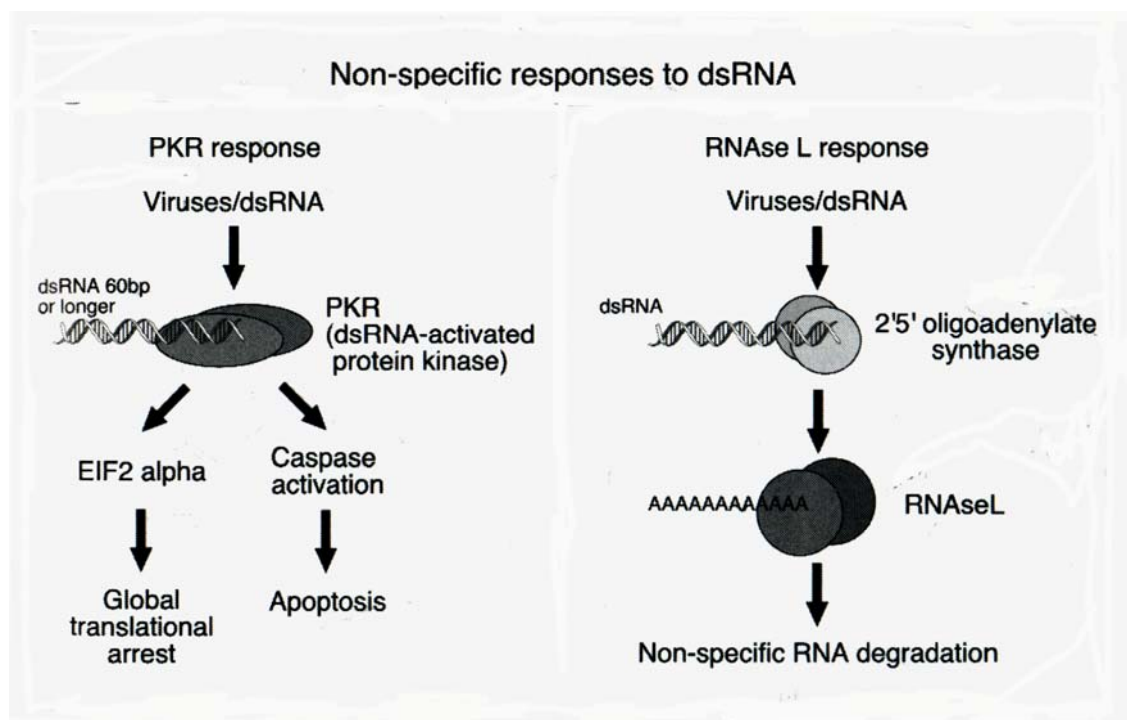
4.1.2 Mammalian system

Practical use of RNAi in mammalian experiments was delayed because the long dsRNA used in plants and invertebrates triggered cell death pathway. In most types of mammalian cells, dsRNA longer than 30 nt triggers dsRNA-activated protein kinase (PKR) and 2',5'-oligoadenylate synthetase (2',5'-AS). These two responses leading to blockage of protein systhesis initiation by phosphorylation and inactivation of the translation factor eIF2a and to RNase L mediated RNA degradation,

respectively (Figure 20) (Kumar and Carmichael, 1998). These pathways are common to the interferon response which is ubiquitous in mammalian cell with the exception of oocytes (Svoboda et al., 2000) and early embryonic cells (Wianny and Zernicka-Goetz, 2000; Billy et al., 2001). With the initial demonstration that short RNA duplexes, siRNAs (~23 nt), could be used to mediate a gene silencing effect in mammalian cells (Elbashir et al., 2001a; 2001b). This finding triggered many studies using RNAi in mammalian cells either in cultured cell (Elbashir et al., 2001a; 2001b; Elbashir et al., 2002; Bridge et al., 2003; Czauderna et al., 2003), or whole animals (Hasuwa et al., 2002; Shinagawa and Ishii, 2003; Stein et al., 2003).
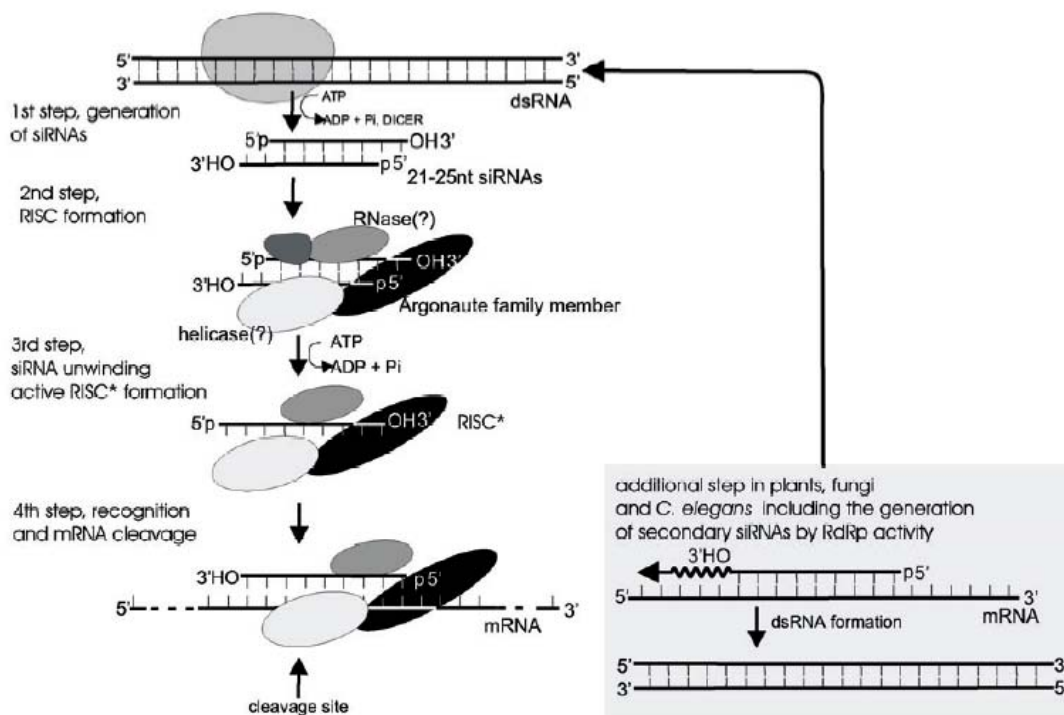
Figure 19. Schematic representation of four-step gene silencing pathway.

dsRNA, double-stranded RNA; siRNA, small interfering RNA; RISC, RNAi-induced silencing complex; RdRp, RNA-dependent RNA polymerase. (Engelke, 2004)

Figure 20. Non-specific responses to dsRNA in mammalian systems.

(Engelke, 2004)