Chapter I

1 A Matter of Death

Like most profound insights, the fact that life cannot exist without death is very simple and almost disappointingly banal. Nonetheless, death is a fundamental part of evolution since it allows the introduction of genetic variance through reproduction while disallowing the persistence of non-advantageous mutations. In a multicellular organism the evolutionary argument of genetic variability is replaced by the need for the "common good". For example, introduction of genetic variability in even a small compartment, and likewise, unchecked growth, into a healthy organism will almost invariably be detrimental to the whole organism. Therefore, cells usually enter a state of post-mitotic dormancy, eventually followed by senescence. Nonetheless, in order to deal with aberrant cells that have escaped this surveillance, or because of the need to shape tissues during development or after injury, a mechanism of cellular death evolved. On a cellular level, two forms of cell death are generally distinguished: necrosis and apoptosis (Kerr et al., 1972).

1.1 Necrosis

Necrosis, also sometimes referred to as accidental cell death, usually is brought about by non-specific, non-physiological insults like injury or gross environmental perturbations. It leads to inflammatory responses and the immigration of phagocytic cells into the affected tissue, which remove the necrotic cells.

1.1.1 Morphological Features of Necrosis

Necrotic cells and their organelles swell up and become enlarged; vacuoles appear throughout the cytoplasm. Very soon after the initial insult the plasma membrane integrity breaks down and the cellular and organelle contents are spilled out. Chromosomal DNA of cells undergoing necrosis stays largely intact and does not condense. The uncontrolled spill of cellular content out of the ruptured cells into the environs leads to the recruitment of phagocytic cells and the subsequent release of inflammatory molecules.

1.2 Apoptosis

The word apoptosis means "falling leaves" and is derived from the Greek αποπτωσιε which was first used by Hippocrates (~460-370BC) in a medical context (Esposti, 1998). It was introduced into scientific literature only recently to describe a form of cell death morphologically distinct from necrosis (Kerr et al., 1972). The term apoptosis is sometimes used synonymously with "Programmed Cell Death" to indicate that it is a genetically inscribed process whose components are conserved in animals. Apoptosis sometimes is also referred to as cell suicide. In stark contrast to necrosis, apoptosis does not lead to inflammation, and neighbouring, unaffected cells or tissue phagocytes take up apoptotic residues.

Apoptosis is a tightly regulated process that removes unwanted or unnecessary cells from an organism. It plays an important role in tissue homeostasis since it results in extensive deletion of cells with little tissue disruption. Dysregulation of apoptosis has severe effects on the organism: Too little apoptosis leads to the development of cancerous cells or autoimmune diseases; too much results in the removal of crucial cells and therefore damages the development of the organism. Examples of apoptosis

include the removal of the interdigital webs during embryogenesis, the removal of activated T-cells after an immune response, thymocyte maturation in the thymus or cell death of neurons during neural development (Jacobson et al., 1997; Pettmann and Henderson, 1998).

Apoptosis is induced by specific stimuli. Physiologic inducers of apoptosis include growth factor withdrawal, certain cytokines, glucocorticoid hormones and some cell surface proteins. Non-physiological insults such as DNA damage, ultraviolet radiation or hypoxia also induce apoptotic cell death.

1.2.1 Morphological Features of Apoptosis

Early during apoptosis, the cytoplasm and cytoplasmic organelles condense and the cell starts shrinking; the asymmetry of the plasma membrane becomes compromised by the externalization of phosphatidylserine, which marks the cell as apoptotic to its neighbours. The cell rounds up and detaches from its neighbours or the substratum. Later, the chromatin condenses and aggregates on the nuclear membrane. The nuclear membrane becomes abnormally convoluted and eventually breaks down completely. Finally, the DNA is cleaved into distinct nucleosomal fragments of about 200bp; the integrity of the plasma membrane breaks down, as the membrane starts blebbing and the cell disintegrates into distinct apoptotic bodies. However, no leakage of cellular contents occurs. These apoptotic bodies contain shrunken organelles and fragmented pieces of chromosomal DNA. They are taken up by neighbouring cells and tissue phagocytes where they are digested in phagosomes.

1.3 Apoptosis and Necrosis: A continuum?

Although, theoretically, most cases of apoptosis and necrosis are easily distinguished from each other, recent research shows that reality is ultimately more complicated. The gross environmental perturbation of hypoxia, for example, induces neurons to undergo apoptosis. Furthermore, in some cases, a class of apoptotic molecules, the caspases (see below) are not activated, yet the cells are still considered apoptotic (Lorenzo et al., 1999). Also, some programmed cell deaths in neurons are executed by a mechanism involving autophagy (Klionsky and Emr, 2000). On the other hand, in some necrotic cells, caspases seem to be activated and Bcl-2 is able to inhibit both apoptosis and necrosis (Kitanaka and Kuchino, 1999; Tsujimoto et al., 1997). The description of forms of programmed cell death that share aspects of both apoptosis and necrosis, termed "oncosis" (Levin, 1998; Majno and Joris, 1995; Shirai, 1999) and "paraptosis" (Sperandio et al., 2000), respectively, suggests that "pure" apoptosis and "pure" necrosis lie on opposite ends of a wide spectrum of various forms of cell death. Further evidence supporting this theory comes from the study of ischemic brain lesions that show signs of necrosis in the center and apoptosis in the outlying regions of the lesions (Graham and Chen, 2001).

2 The Molecular Components of Apoptosis

Real progress in understanding the molecular basis of apoptosis has only been made in the last decade of the 20th century, twenty years after the initial description of apoptosis. A flurry of discoveries since then has unearthed the underlying mechanisms. In the initial phase, mutational screening in the nematode *C. elegans* has identified several *cell death abnormal* (CED) genes that are involved in apoptotic regulation. Of these, only three play a direct role in the execution of apoptosis: CED-3, CED-4 and CED-9 form a complex and execute apoptosis (Metzstein et al., 1998). Mammalian and even Drosophila counterparts have been identified through sequence homologies and, ultimately, share functional similarities.

2.1 Death Receptors

Death receptors (DR) form a subfamily of the Tumor Necrosis Factor Receptor/Nerve Growth Factor Receptor (TNFR/NGFR) superfamily of cytokine receptors (Ashkenazi and Dixit, 1999; Ashkenazi and Dixit, 1998). The DR subfamily is distinguished by the presence of a homotypic protein-protein interaction domain in its cytoplasmic tail, termed death domain (DD) (Tartaglia et al., 1993), that directly transmits the death signal to the apoptotic machinery. To date, six DR have been identified (Fig. 1). In addition, the p75 NGFR also contains a DD that is essential for the transmission of its apoptotic signal. Fas and DR3 play a crucial role in controlling apoptosis in the immune system, whereas the functions of DR4, 5 and 6 remain unknown.

2.2 Bcl-2 Family

The Bcl-2 family of apoptotic regulators is named after the gene *bcl-2*, which is activated by chromosome translocation in follicular lymphoma (Bakhshi et al., 1985; Tsujimoto et al., 1984). To date, at least 15 family members have been identified (Fig. 2). All members posses at least one of four conserved motifs known as Bcl-2 homology domain BH1 to BH4 and frequently a short transmembrane domain (Adams and Cory, 1998). The transmembrane domain targets Bcl-2 proteins to specific cellular compartments, most importantly to the endoplasmatic reticulum and the outer mitochondrial membrane.

The family is divided into anti- and pro-apoptotic members. Anti-apoptotic members contain at least BH1 and BH2 and inhibit apoptosis caused by a variety of stimuli. The pro-apoptotic group can be further subdivided into the Bax-subfamily and the BH3-subfamily. All pro-apoptotic family members contain the BH3 domain, but the BH3-subfamily members contain little else.

Pro- and anti-apoptotic family members hetero- and homodimerize with each other by means of BH domain interactions (Chittenden et al., 1995; Kelekar and Thompson, 1998; Yin et al., 1994). The α-helices of the BH1, BH2 and BH3 domains form a hydrophobic pocket to which another BH3 helix binds (Muchmore et al., 1996; Sattler et al., 1997). These two structures are therefore also referred to as receptor and ligand, respectively. This ability to heterodimerize means that pro-and anti-apoptotic molecules effectively titrate each other for control of the survival status of the cell (Oltavai et al., 1993). Thus, anti-apoptotic Bcl-2 family members keep pro-apoptotic Bcl-2 proteins from forming pro-apoptotic heteromers that otherwise activate the apoptotic program.

2.3 Apaf-1 Family

Apoptotic protease activating factor-1 (APAF) has originally been described as a cytoplasmic homologue of the *C. elegans* protein CED-4 that promotes apoptosis (Zou et al., 1997). Since then, several other homologues have been identified, most notably NOD1 (Imai et al., 1999; Inohara et al., 1999; Ogura et al., 2001). However, the exact relationship of these other family members to apoptosis is not clearly established.

Apaf-1 is a cytoplasmic protein of ~130kD that consists of a N-terminal caspase-recruitment domain (CARD) (Hofmann et al., 1997), followed by a CED-4 homology domain containing a nucleotide binding domain (NBD, also called A-box) and a P-loop motif (also called B-box). The C-terminus is made up of several WD40-domains that are responsible for cytochrome c binding. It plays a pivotal role in integrating and transmitting apoptotic signals and activating the effector machinery of apoptosis.

2.4 caspases

The effector molecules of apoptosis, caspases are a family of cysteine proteases with aspartate specificity (cysteine aspartases) (Kumar and Colussi, 1999; Thornberry and Lazebnik, 1998). Caspases are synthesized as single chain pro-enzymes that undergo activation by proteolysis. Upon activation the zymogen is cleaved into a large p20 and a small p10 subunit. The active enzyme exists as a heterodimer of two p20 and two p10 subunits and thus displays two active sites (Rotonda et al., 1996; Walker et al., 1994; Wilson et al., 1994). The active site of caspases consists of a Cys-His catalytic dyad; substrate specificity is conferred through substrate binding

pockets S1-S4, with S1 accommodating Asp, while S3 accepts any amino acid but prefers Glu (Stennicke and Salvesen, 1998).

To date, 15 caspases have been identified (Fig. 3) (Thornberry and Lazebnik, 1998) which are classified into two subgroups by means of the length of an N-terminal pro-domain or caspase recruitment domain (CARD). Upon proteolytic activation, the CARD is clipped off, except in the case of caspase-9, where no pro-domain processing occurs (Hengartner, 2000). Long pro-domain caspases are further divided into two groups: Caspases-1, -4, -5, which play a more prominent role in cytokine activation than in apoptosis; caspases-2, -8, -9, -10, which are called initiator, or signaling, caspases since they integrate and transduce various apoptotic signals to the effector machinery by means of proteolytic activation. The short pro-domain, or effector/ executor, caspases –3, -6, -7 become activated by the initiator caspases and are able to activate themselves, as well as the initiator caspases, thus amplifying the apoptotic signal. They are also directly responsible for cleavage of cellular proteins that lead to apoptotic morphology.

Caspase substrates are divided into four distinct groups (Villa et al., 1997): Class I substrates become activated upon cleavage, Class II substrates become inactivated, Class III substrates alter their assembly/disassembly properties and Class IV substrates undergo cleavage with unknown consequences.

2.5 DNases

Activation of caspases commits the cells to die. However, to ensure ultimate and non-reversible cell disassembly, chromosomal DNA must be cut and degraded. The protein responsible for apoptotic DNA digestion is

called DNA Fragmentation Factor/caspase-activated DNase (DFF/CAD) (Enari et al., 1998; Liu et al., 1997). In non-apoptotic cells, CAD is bound to its inhibitor ICAD. However, upon induction of apoptosis, caspase-3 cleaves ICAD and CAD is released (Liu et al., 1998; Sakahira et al., 1998). Activated CAD is able to bind Histone H1 that directs it to the linker region between nucleosomes where CAD cleaves both DNA strands (Liu et al., 1999).

2.6 Cytochrome C

While searching for cytoplasmic factors capable of inducing apoptosis, the group of X. Wang identified three Apaf molecules. Apaf-3 turned out to be caspase-9 (Li et al., 1997), while, most surprisingly, Apaf-2 was identified as cytochrome c (Liu et al., 1996). The known function of cytochrome c is to transfer electrons from the cytochrome-b-c1 complex to the cytochrome oxidase complex in the respiratory chain (Salemme, 1977). Now, a second function of cytochrome c is observed. When released into the cytosol, cytochrome c is capable of inducing apoptosis by binding to Apaf-1 and thereby activating it. cytochrome c release from the mitochondria is recognized as a hallmark event during apoptosis and is increasingly used as diagnostic marker for apoptosis.

2.7 Inhibitors of Apoptosis

The Inhibitor of Apoptosis (IAP) protein family to date consists of seven members that introduce yet another regulatory step to the apoptotic signaling pathways (Goyal, 2001). IAPs have first been described in baculovirus and have later been found in other organisms. They consist of one to three baculoviral IAP repeat (BIR) domains. In addition, some IAPs

also contain a C-terminal RING domain, a protein motif found in ubiquitin ligases. During apoptosis they bind to active caspases by means of the BIR-domain, thereby inactivating them, while the RING-domain targets the IAP-caspase complex for ubiquitin-dependent degradation. The cytoplasmic IAPs are also cleaved by caspases. The resulting cleavage products, however, have differing specificity and directly inhibit caspases -3, -7 and -9 with varying degrees of effectiveness (Chai et al., 2001; Deveraux et al., 1999; Deveraux et al., 1997; Harlin et al., 2001; Huang et al., 2001; Riedl et al., 2001; Roy et al., 1997; Suzuki et al., 2001). Thus, IAPs serve as additional regulators of the execution phase of apoptosis.

Interestingly, during apoptosis, along with cytochrome c, another molecule is released from mitochondria. Smac/DIABLO (Du et al., 2000) (Verhagen et al., 2000) directly binds to the BIR-domain of IAPs (Liu et al., 2000) and thus inhibits their ability to bind and inhibit activated caspases.

2.8 Other molecules

A plethora of other molecules has been identified that play an important role in regulation of apoptosis. For the sake of brevity, only some of these are listed and their functions briefly explained.

Acinus: Acinus (Sahara et al., 1999) is a mainly nuclear protein that is activated by cleavage during apoptosis and causes chromatin condensation without DNA fragmentation.

Apoptosis Inducing Factor (AIF): A mitochondrial flavoprotein that induces caspase-independent apoptosis and leads to DNA cleavage into large fragments (Daugas et al., 2000; Susin et al., 1999).

Heat Shock Proteins (HSP): Several HSPs are reported to bind caspases and/or Apaf-1, thereby regulating caspase activation (Beere et al., 2000; Bruey et al., 2000; Garrido et al., 1999; Pandey et al., 2000).

3 Apoptotic Signaling Pathways

Apoptotic signaling pathways are divided into two parts: an initiator part that employs specific signaling routes for each apoptotic stimulus and a common effector pathway. The exact signaling mechanisms for most death inducers, such as staurosporine, dexamethasone, UV-irradiation etc. are still elusive. All initiator routes, however, converge onto the effector part. The effector part comprises the effector caspases, apoptotic DNases and all other molecules that actively play a role in killing the cell. The following is a brief overview of the major apoptotic pathways (Fig. 4).

3.1 Mitochondria-dependent death

Perhaps the biggest surprise of apoptosis research is the finding that an organelle that was thought to be well understood acquires a prominent new role. Apart from being the energy generator of the cell, mitochondria play a pivotal role in regulating apoptosis (Fig. 5) (Crompton, 2000; Kroemer and Reed, 2000).

During apoptosis the outer mitochondrial membrane potential $\Delta\Psi m$ breaks down and the energy generating function of the mitochondrium ceases (Ankarcrona et al., 1995; Castedo et al., 1996; Deckwerth and Johnson, 1993; Petit et al., 1995; Zamzami et al., 1995; Zamzami et al., 1995). This breakdown in $\Delta\Psi m$ is caused by a change in membrane permeability that leads to the release of cytochrome c, AIF, Smac/DIABLO and several caspases (and presumably even more molecules) from the mitochondrial intermembrane space into the cytosol (Daugas et al., 2000; Du et al., 2000; Liu et al., 1996; Samali et al., 1999; Susin et al., 1999). How the mitochondrial permeability transition (PT) is brought about is the subject of intense research.

Many Bcl-2 family members reside in the outer mitochondrial membrane oriented towards the cytosol by means of an N-terminal transmembrane domain. Experiments show that Bax, Bcl-2 and Bcl-x_L form ion channels when added to synthetic membranes (Antonsson et al., 1997; Minn et al., 1997; Schendel et al., 1997; Schlesinger et al., 1997). Indeed, these pores resemble pores that are formed by certain bacterial toxins such as diphtheria toxin. However, these pores are too small for passage of macromolecules; thus, PT might only indirectly be brought about by the pore forming ability of Bcl-2 family proteins.

Alternatively, a large conductance channel, the PT pore, has been proposed to regulate PT (Marzo et al., 1998). The structure and components of this PT pore remain largely undefined, but its constituents reside both in the outer and the inner mitochondrial membrane. On the inner membrane, Adenine Nucleotide Translocator (ANT) (Marzo et al., 1998), and on the outer membrane the Voltage-dependent Anion Channel (VDAC) are members of this pore (Narita et al., 1998; Shimizu et al., 1999). These two molecules act in concert, thus creating a channel crossing both mitochondrial membranes that is permeable for molecules ≤13kD.

The PT pore in turn is regulated by Bcl-2 family proteins. VDAC acts as a receptor for Bax, thereby recruiting it to the mitochondria. However, the exact mechanism how Bax induces PT and release of apoptogenic molecules remains unclear. The channel created by ANT-VDAC allows only very small molecules like cytochrome c to pass, but not larger ones such as AIF. Hyperosmolarity of the mitochondrial matrix, due to the higly impermeable inner membrane that keeps the components of the mitochondrial metabolic pathways and most ions inside. Activation of the PT pore leads to volume dysregulation and rupture of the membrane (Crompton and Costi, 1988;

Marzo et al., 1998). Membrane rupture would account for release of macromolecules. However, cells whose mitochondria are swollen and ruptured are very likely to die by a necrotic mechanism (Crompton, 1999).

It remains unclear and is the subject of intense research how all these events fit together. Even the most fundamental question whether PT is brought about by the formation of an ion channel, a pore or simply through osmolar mechanisms is still a mystery.

The events following the mitochondrial step are much better understood. Once cytochrome c is released it binds to Apaf-1 (Li et al., 1997). Apaf-1 resides in the cytosol of apoptotic cells as part of a large protein complex called the apoptosome (Cain et al., 2000; Zou et al., 1999). The exact configuration of inactive Apaf-1 is unclear. The active apoptosome complex has a molecular weight of ~700kD; a second Apaf-1 containg complex of ~1.4MD is described (Cain et al., 2000). However, this 'inactive' apoptosome is probably just an aggregation of misfolded proteins, including improperly activated Apaf-1. The other components of the apoptosome complex are mostly unknown, but several heat shock proteins are implicated (Beere et al., 2000; Bruey et al., 2000; Pandey et al., 2000; Saleh et al., 2000).

Cytochrome c activates Apaf-1 by binding to its WD40 domains (Adrain et al., 1999; Benedict et al., 2000; Srinivasula et al., 1998), which leads to oligomerization of Apaf-1 accompanied by hydrolysis of ATP/dATP (Li et al., 1997). Oligomerization of Apaf-1 brings pro-caspase-9 molecules bound to Apaf-1 via CARD-CARD interactions into close proximity to each other (Hofmann et al., 1997; Qin et al., 1999; Zhou et al., 1999), leading to autocatalytic cross activation of paired caspase-9 by proteolysis. Once caspase-9 is activated, it directly cleaves and activates its

downstream substrate pro-caspase-3 (Li et al., 1997). Caspase-3 is the first caspase of the effector arm of apoptosis and directly cleaves other effector caspases as well as initiator caspases (Slee et al., 1999) thus amplifying the apoptotic signal. The effector caspases cleave cellular substrates such as ICAD, nuclear lamins or Poly-ADP-Ribose Polymerase (PARP, an important marker for detection of apoptosis) (Villa et al., 1997).

As noted before, along with cytochrome c, several other pro-apoptotic molecules are released from the mitochondria during apoptosis. Among those is Smac/DIABLO which disables IAPs. Thus, mitochondria not only release caspase-activating, but also caspase-inhibitor deactivating factors.

3.2 Death Receptor Pathway

The signaling pathways activated by the prototype DR Fas (DR1) and TNFR1 (DR2) are the first apoptotic pathways to be understood at the molecular level (Fig. 6) (Ashkenazi and Dixit, 1998). These pathways therefore serve as paradigms for DR signaling and will be described below. Other DR show slightly different behaviour.

DR recruit adapter molecules via DD-DD interactions (Boldin et al., 1995; Huang et al., 1996); Fas binds Fas-associated DD-protein (FADD) (Boldin et al., 1995; Chinnaiyan et al., 1995), whereas TNFR1 binds TNFR-associated DD-protein (TRADD) (Hsu et al., 1995) which in turn binds FADD (Hsu et al., 1996; Varfolomeev et al., 1996). FADD also contains a death effector domain (DED) with which it binds the DED of pro-caspase-8 (Boldin et al., 1996; Muzio et al., 1996).

The DR pathway is activated by binding of the trimeric ligand to its trimeric receptor and the formation of the death-inducing signaling complex (DISC). The DISC contains the DR, and the respective adapter molecules

FADD and TRADD (Muzio et al., 1996). DISC formation leads to the recruitment of caspase-8 to FADD. This brings the caspase-8 zymogens into close proximity and they auto-catalytically cross-activate each other (the induced proximity model of caspase activation) (Muzio et al., 1998). Caspase-8 connects directly to the effector arm by activating caspase-3 (Stennicke et al., 1998). Caspase-8 also cleaves Bid (Li et al., 1998; Luo et al., 1998). Cleaved Bid in turn binds strongly to Bax, thereby activating it (Desagher et al., 1999; Eskes et al., 2000), leading to cytochrome c release from mitochondria, thus amplifying the DR-mediated death signal.

Recently, cells have been divided into two groups by their properties of DR-mediated apoptosis. Type I cells contain high levels of DISC activity and are able to undergo apoptosis without the mitochondrial amplification loop, whereas Type II cells show low DISC levels and need the Bid-mediated mitochondrial amplification to efficiently undergo receptor-mediated apoptosis (Scaffidi et al., 1998).

While most DR solely transmit a signal for cell death, some have at least dual function. Some DR (e.g. TNFRI, DR3) also recruit other adapter proteins like TNFR-associated factors (TRAFs) (Hsu et al., 1996; Rothe et al., 1995; Shu et al., 1996; Takeuchi et al., 1996; Wang et al., 1998) or Receptor Interacting Protein (RIP) (Hsu et al., 1996; Stanger et al., 1995) that connect the receptor to the NF-κB signaling pathway. Depending on cellular context TRAFs also activate the c-jun N-terminal kinase (JNK) pathway. While activation of the transcription factor NF-κB is generally associated with anti-apoptotic activity, JNK and related stress-induced signaling pathways induce both pro- and anti-apoptotic genes (Liu et al., 1996). Pro-apoptotic genes induced by these pathways likely act on the mitochondrial death pathway.

3.3 The C. elegans death machinery

Two forms of apoptosis are generally distinguished: Intrinsic and instructive apoptosis. Intrinsic apoptosis is a genetically inscribed process that commits a cell to apoptosis at a certain developmental stage. Instructive apoptosis occurs upon stimulation by some extra-/intracellular signal, e.g. DR ligation. During development of the nematode *C. elegans* 1090 cells are generated, of which 131 die by intrinsic apoptosis (Metzstein et al., 1998). In the germline, apoptosis occurs by an instructive mechanism (Gumienny et al., 1999).

Several genes have been identified that regulate apoptosis in *C. elegans*. The core machinery consists of only three proteins: CED-9 is an anti-apototic member of the Bcl-2 family (Hengartner and Horvitz, 1994); CED-4 is an Apaf-1 homologue (Yuan and Horvitz, 1992), but does not contain the WD-40 domain; CED-3 is the caspase and is most homologous to caspase-9 (Yuan et al., 1993). Other proteins, like CED-1 (Zhou et al., 2001), CED-5 (Wu and Horvitz, 1998) or CED-7 (Wu and Horvitz, 1998) regulate engulfment of apoptotic corpses and their subsequent degradation. Egl-1 is a BH-3-only protein that negatively regulates CED-9 (Conradt and Horvitz, 1998). Egl-1 in turn is transcriptionally regulated in some cells by Ces-1 and Ces-2 (Metzstein et al., 1996; Metzstein and Horvitz, 1999).

The core machinery is very simple and probably an evolutionary precursor to the much more sophisticated mammalian death machinery. CED-3, CED-4 and CED-9 interact and bind to each other and form the *C. elegans* analogue of the apoptosome (Fig. 7) (Chinnaiyan et al., 1997; Wu et al., 1997). Just like Apaf-1, CED-4 oligomerizes and activates CED-3 (Yang

et al., 1998), but without the need for cytochrome c activation. The activation of the caspase is counteracted by CED-9, which binds CED-4 and inactivates it. Inhibition of CED-4 is relieved if Egl-1 disrupts CED-9 binding to CED-4 by inserting its BH-3 domain into CED-9 (del Peso et al., 2000; del Peso et al., 1998). In a cell that is not doomed to die, Egl-1 is transcriptionally silenced. In contrast, a cell that is committed to apoptosis upregulates Egl-1, thereby activating CED-4 and CED-3.

4 Huntingtons Disease

4.1 Disease Phenotype

Huntingtons Disease (HD) is an autosomal-dominant neurodegenerative disorder (Lacour, 1990) that primarily affects the medium-size spiny neurons of the striatum (Kowall et al., 1987; Vonsattel et al., 1985). It is caused by an expansion of CAG trinucleotides in the gene IT15 which results in an expansion of a stretch of glutamines in the encoded Huntingtin (Htt) protein (Huntington's Disease Collaborative Research Group, 1993). The threshold for the occurrence of HD is between 34 and 37 poly-Q residues (Harper, 1999; MacDonald and Gusella, 1996). Expansion of the poly-Q stretch to more than 100 residues leads to juvenile onset HD (Hayden et al., 1985; Nance et al., 1999), showing that disease onset is inversely correlated to the length of the CAG repeat (Gusella et al., 1997; Kremer et al., 1993).

HD is characterized by progressive loss of cells of the striatum and the cerebral cortex. HDs clinical symptoms include chorea, dementia and personality change (Ross et al., 1997). Symptoms typically begin in mid-adulthood and progressively worsen until death ~ 18 years from onset (Beighton and Hayden, 1981).

4.2 Molecular basis of HD

The Htt protein is a cytoplasmic protein of 350kD (Fig. 8) with no homologies to other proteins. Its expression is widespread and not restricted to the nervous system (Sharp and Ross, 1996; Trottier et al., 1995). Htt shows no easily recognizable domains. However, it contains a stretch of glutamine residues in its N-terminal part (15-36 for the wild-type protein,

≥40 to >120 for the mutated form), followed by a proline-rich region. In addition, Htt contains several putative regulatory motifs like a conserved consensus site for phosphorylation by the Akt protein kinase at Ser421 and several caspase-cleavage sites, most importantly two caspase-3 sites at Asp513 and Asp530. Of these two sites, however, only Asp513 is cleaved by caspase-3 *in vitro* (Wellington et al., 1998; Wellington et al., 2000).

Poly-Q expansion is a gain-of-function, rather than a loss-of-function event (MacDonald and Gusella, 1996). This is demonstrated in studies with mice transgenic for exon 1 of the Htt gene. Exon 1 comprises of the poly-Q repeat and the proline rich region. Mice with wild-type (wt) exon 1 remained healthy. However, mice with 118-156 CAG-repeats developed neurological symptoms (Bates et al., 1997; White et al., 1997). Since these mice also express the wtHtt from their own genome, this demonstrates that the mutant (mu) transgene imposes a new dominant phenotype.

In HD, cell death is correlated with the occurrence of intranuclear inclusion bodies (IIN) that stain positive for ubiquitin (Becher et al., 1998; Cooper et al., 1998; Davies et al., 1997; DiFiglia et al., 1997; Gourfinkel-An et al., 1998; Sieradzan et al., 1999). These IIN are formed by the N-terminal caspase cleavage product of Htt containing the poly-Q stretch. Poly-Q polypeptides have been reported to self-aggregate and form insoluble precipitates (Perutz, 1999). Even in the context of larger proteins poly-Q retains the ability to aggregate. Indeed, muHtt has the ability to form amyloid-like fibers and aggregates *in vitro* and *in vivo* (Scherzinger et al., 1997; Scherzinger et al., 1999).

Initially, IIN have been used as a diagnostic marker for HD and were thought to be causally involved in HD pathology (DiFiglia et al., 1997). However, Saudou et. al. show that cell death does not correlate with

inclusion formation (Saudou et al., 1998). In this study, cultured striatal neurons die by an apoptotic mechanism upon transfection of muHtt. Suppression of nuclear localization of muHtt suppresses inclusion formation and neurodegeneration. However, when conditions are chosen that only suppress inclusion formation, muHtt-induced cell death is increased. This clearly demonstrates that IIN are most likely just a side effect of HD and represent a cellular coping mechanism to degrade insoluble aggregates via ubiquitination and degradation by the proteasome (Kim and Tanzi, 1998). Therefore, inclusions may reflect a cellular protection mechanism against muHtt.

Further evidence for this comes from studies with a yeast artificial chromosome (YAC) mouse model. Mice, expressing a YAC encoding FL-muHtt in the context of the complete gene including upstream regulatory sequences develop neurodegeneration even in the absence of aggregates (Hodgson et al., 1999).

Nonetheless, caspase-cleavage of muHtt is a crucial event in disease pathogenesis. Experiments show that mutation of the caspase-cleavage sites at D513, D530 and D586 reduces muHtt cytotoxicity in a cellular model (Wellington et al., 2000). The importance of the cleavage sites for disease pathogenesis *in vivo* by the generation of cleavage site knock-in mutant mice has not yet been assessed. Moreover, caspase inhibitors also reduce muHtt toxicity (Kim et al., 1999). Consistent with the hypothesis of caspase involvement are the findings that lymphoblasts from HD patients show increased activity of caspases -3 and -9 (Sawa et al., 1999) and that expression of a dominant-negative form of caspase-1 in the background of a Htt transgenic mouse model slows disease progression (Ona et al., 1999). The involvement of caspase-1 is further supported by data that demonstrates

nuclear muHtt transcriptionally upregulates caspase-1 (Li et al., 2000). In addition, caspase-8 has been shown to be recruited to and activated by long poly-Q peptides (Sanchez et al., 1999).

Whether caspase activation occurs by direct or indirect mechanisms is unclear. Current thinking favours an indirect model in which muHtt interferes fatally with the transcriptional activity of the cell (Cha, 2000). Several proteins containg Q-rich regions are themselves transcription factors and the poly-Q stretch can act as a transcriptional activator (Gerber et al., 1994). Indeed, the expanded poly-Q stretch in muHtt resembles similar regions in a variety of transcription factors such as N-oct3, TATA-binding protein (TBP) or CREB-binding protein (CBP). Dysregulation of transcription could possibly also account for the observed cellular specificity and the delayed disease onset.

Recently it has been reported that muHtt interacts with the transcription factors CA150 (Holbert et al., 2001), p53 and CBP (Steffan et al., 2000) and represses CBP-mediated transcription (Nucifora et al., 2001). Thus, muHtt alters the transcriptional activity of the cell, either directly by binding DNA, or indirectly by binding to various transcription factors. This could conceivably lead to downregulation of pro-survival genes, such as neurotrophic factors (Finkbeiner, 2000; Finkbeiner et al., 1997), or to up-regulation of pro-apoptotic genes.

Nonetheless, the mechanisms by which muHtt delivers the initial toxic insult that leads to muHtt cleavage and activates the apoptotic effector machinery still remain elusive.

4.3 The Function of wtHtt

Much less is known about wtHtt and its function remains unclear. The only functional evidence comes from studies of mice with a targeted disruption of the Htt gene. These mice die at embryonic day 7.5 with signs of increased apoptosis in the developing ectoderm (Duyao et al., 1995; Nasir et al., 1995; Zeitlin et al., 1995). These data suggest that Htt plays an important anti-apoptotic role during embryonic development that is not restricted to the nervous system. The phenotype of Htt -/- mice can be rescued by muHtt (Hodgson et al., 1999; Hodgson et al., 1996), indicating that muHtt retains some of the function of the wild type protein.

Further evidence supporting a broader role for Htt that is not restricted to function in neurons is demonstrated in studies with embryonic stem cells from Htt -/- mice. These cells are viable and can be turned into mature neurons in cell culture experiments (Metzler et al., 1999), indicating that Htt is not required for neuronal development. Moreover, recent data demonstrate a role for Htt in hematopoiesis by aiding survival of hematopoietic progenitors (Metzler et al., 2000).

In addition to this pro-survival function, Htt has been shown to be upregulated in response to iron depletion and might play a role in general iron metabolism (Hilditch-Maguire et al., 2000). In this study, cellular organelles of Htt -/- mice are investigated for abnormalities. Defects are found in perinuclear recycling of endosomes as demonstrated by impairment of transferrin receptor recycling. Additionally, Htt is found to be upregulated when the iron-chelator deferoxamine mesylate is administered. Nonetheless, no iron-responsive element (IRE) is present in the non-coding regions of the IT15 gene.

A role in membrane trafficking and cellular morphogenesis has been described as a third potential function of Htt (Hattula and Peranen, 2000). In this work, Rab8, a protein involved in controling polarized membrane transport, binds to FIP-2, a protein with similarities to NEMO, a protein involved in the NF-κB pathway, by yeast two-hybrid screen. Likewise, FIP-2 is shown to bind Htt and thus Rab8.

Both of these studies, however, only offer circumstantial evidence for the Htt function they propose and fail to provide an explanation to reconcile their findings and the apoptotic phenotype observed in Htt -/- mice.

It is conceivable that a huge protein like Htt performs all three putative functions. Different, still unidentified, domains of the protein might take part in different cellular activities. However, no direct evidence for either functionality has been produced thus far. Therefore, the observations described above could possibly also be just manifestations of a single (as yet unknown) functionality.

4.3 Other triplet expansion diseases

HD belongs to a class of neurodegenerative diseases that is defined by pathogenic expansion of CAG nucleotide triplets (Gusella et al., 1997; Lunkes and Mandel, 1997). They include Dentaturubral Pallidolysian Atrophy (DRLPA, Smith disease), Spinobulbar Muscular Atrophy (Kennedy disease), Spinocerebrellar Ataxia-3 (SCA-3, Machado-Joseph disease, MJD), Spinocerebellar Ataxia-6 (SCA-6) and Spinocerebellar Ataxias-1, -2, -7 (SCA-1, -2, -7). The affected proteins are Atrophin (Nagafuchi et al., 1994; Onodera et al., 1995), Androgen Receptor (La Spada et al., 1991), Ataxin-3 (Kawaguchi et al., 1994), Purkinje cell-specific Ca^{2+} channel subunit α_{1A} (Jodice et al., 1997; Riess et al., 1997) and Ataxin-1, -2, -7

(Chung et al., 1993; David et al., 1997; Imbert et al., 1996; Orr et al., 1993; Pulst et al., 1996; Sanpei et al., 1996), respectively.

Like HD, these diseases are caused by a poly-Q expansion in their respective genes . The clinical symptoms resemble each other, including disease onset, chorea and progressive neurodegeneration. Like Htt, expression of these proteins is widespread, although cellular specificity of these diseases is different from HD and, like in HD, only a specific subset of neurons is affected. Little is known about the exact molecular mechanisms that lead to neurodegeneration in each of these diseases.

However, it can be assumed that all triplet expansion diseases induce cell death through means of the expanded poly-Q stretch. Cytotoxicity seems to be modulated by the context of the affected protein and also by the conditions inside the cell in which it is expressed. Most importantly, even poly-Q expansions in proteins not usually carrying poly-Q stretches leads to a disease phenotype similar to the ones described above (Perutz, 1999). This has been clearly demonstrated by the generation of mice transgenic for a 146 poly-Q stretch in hypoxanthine phosphoribosyl transferase, that develop chorea, show IIN and neuronal cell loss (Ordway et al., 1997). How exactly this cellular toxicity is achieved in any of these disease and how the context of the surrounding protein modulates cell specificity is not known.

5 The goals of this study

The most prominent feature of the Htt protein is its ability to induce neurodegeneration by an apoptotic mechanism if the N-terminal poly-Q stretch is pathogenically expanded. This points to a role for muHtt in regulating apoptosis. Furthermore, since Htt -/- mice are not viable and show increased signs of apoptosis in the epiblast, even the unmutated, non-pathogenic form of Htt might play a role in apoptosis.

Therefore, the primary goal of this study is to understand the role of Htt in apoptosis. This work begins under the hypothesis that wtHtt indeed possesses anti-apoptotic function. To test this assumption, evidence needs to be gathered that either supports or rebuts it.

The evidence of the Htt -/- mouse model points strongly to the proposed anti-apoptotic function of wtHtt. In this study a cellular model system is employed that mimicks striatal cells. This system is used to elucidate the apoptotic activity of Htt. The use of an appropriate cellular model is preferable to performing experiments with mice, since the latter involve the creation of several transgenic lines. A cellular model is easily manipulated and allows investigation of several different lines of inquiry. In addition, evidence gatherered in a cellular model needs to be verified by an *in vivo* model.

Having established a role of Htt in apoptosis, the mechanism of Htt function will be investigated. Firstly, the exact point within the apoptotic pathway at which Htts inhibitory activity takes place is going to be determined. This will be achieved by manipulation of the apoptotic pathway itself. Secondly, once the molecular target is identified, the molecular basis for regulation of Htts target will be elucidated. Several molecular biological and biochemical means will be employed to shed light on these mechanisms.

It is, however, unclear whether the proposed role of Htt is (developmentally) regulated or takes place in all tissues at all times. Thus, this question needs to be addressed. To this end and to verify the data from the cellular system, the nematode *C. elegans* will be employed as a convenient Htt-transgenic *in vivo* model. In addition to the simplicity of its apoptotic machinery and the clearly defined fates of every single cell of this organism, the nematode also lacks a Htt homologue which might interfere with the Htt transgene. These studies should prove useful to address questions of function and/or mechanism.

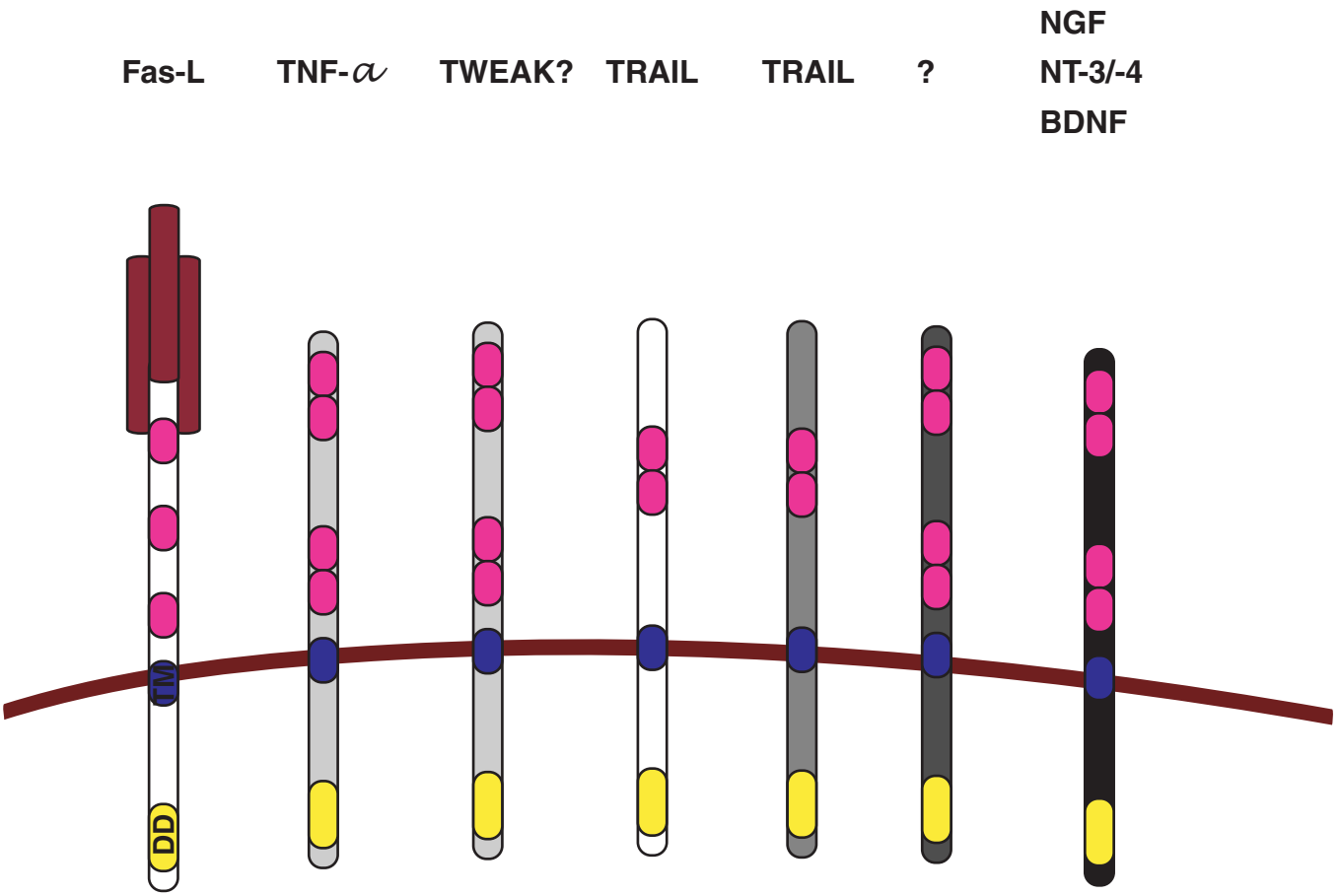
Lastly, while performing all the above mentioned studies, the experiments will be performed in parallel using muHtt. Hopefully, data gathered from these experiments will shed some light into the blackbox that is poly-Q, and namely muHtt, induced cytotoxicity.

6 Figures and Legends

Figure 1

To date, seven Death Receptors have been identified. They are shown here with their respective ligand. All DR contain cysteine-rich repeats of varying lengths in their extracellular domain with which the ligand is bound. In their cytoplasmic domain, DR distinguish themselves from the rest of the TNF/NGFR-superfamily by the presence of a 80aa sequence termed the Death Domain. The DD is a homotypic protein interaction domain, which is essential for apoptotic signaling.

Death Receptors and their Ligands



Cys-rich repeat

Death Domain

Transmembrane Domain

Trimeric Cytokine Ligand

Figure 2

The Bcl-2 family of apoptotic regulators is divided into anti- (green) and pro-apoptotic members (red), representatives of each family are shown here. All family members share at least one of four Bcl-2 homology domains BH1- BH4. Pro-apoptotic molecules are further subdivided into the Bax- and the BH-3-only-subfamilies. The BH1 and BH2 domains are capable of forming small pores upon oligomerization of the proteins. Dimerization occurs through interaction of a 'receptor' domain that is formed by the BH1-BH3 domains with the "ligand", the BH3 domain of another family member. Thus, pro- and anti-apoptotic Bcl-2 proteins form homo- and heteromeric complexes and titrate each other for control of inducing apoptosis.

The Bcl-2 family

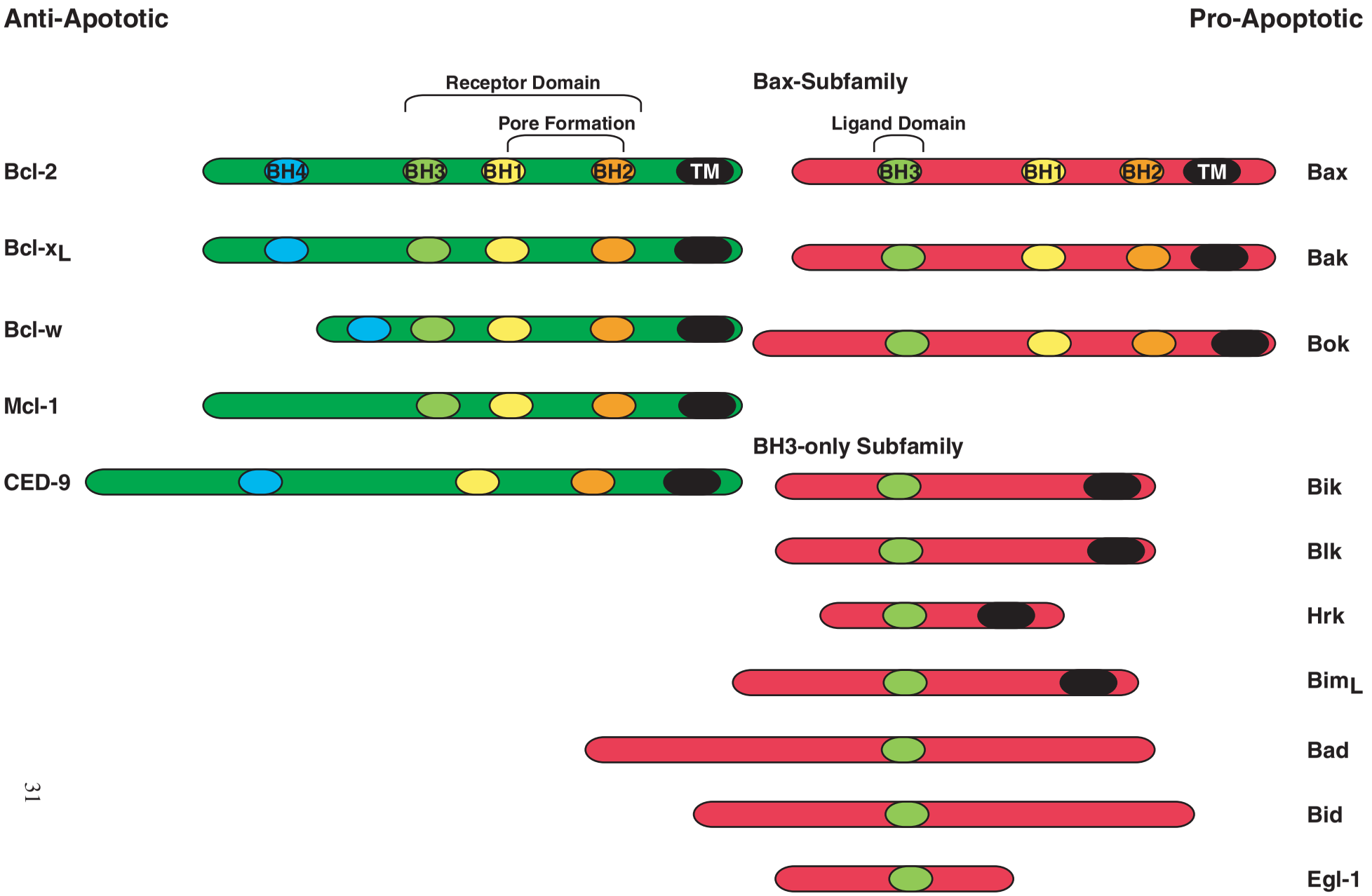


Figure 3

The caspases are the crucial executioner molecules of apoptosis. To date, 15 caspases have been identified, nine of which are classified here. caspases contain an N-terminal signaling domain, or pro-domain, termed caspase-Recruitment Domain, which is a protein-protein interaction domain and very similar to a Death Effector Domain. Sometimes these names are used synonymously. Caspases are divided into two groups, according to the length of their pro-domain. Short pro-domain caspases are called effector caspases, as they are the direct executioners of apoptosis. Long pro-domain caspases are sub-divided into inflammatory caspases, whose role in apoptosis is disputed and who play a more prominent role in inflammation, and initiator caspases who transmit apoptotic signals to the effector caspases through proteolytic activation. As shown, long pro-domain caspases activate themselves via cleavage upon pro-domain mediated dimerization. They also cleave off the pro-domain (except caspase-9) and form the fully active enzyme consisting of heteromers of two p10 and p20 subunits. These molecules then cleave and activate downstream caspases.

The Caspases

Inflammatory Long Pro-domain Caspases

Caspase-1

Caspase-4

Caspase-5

Initiator Caspases

Caspase-8

Caspase-9

Caspase-10

Effector Caspases

Caspase-3

Caspase-7

Caspase-6

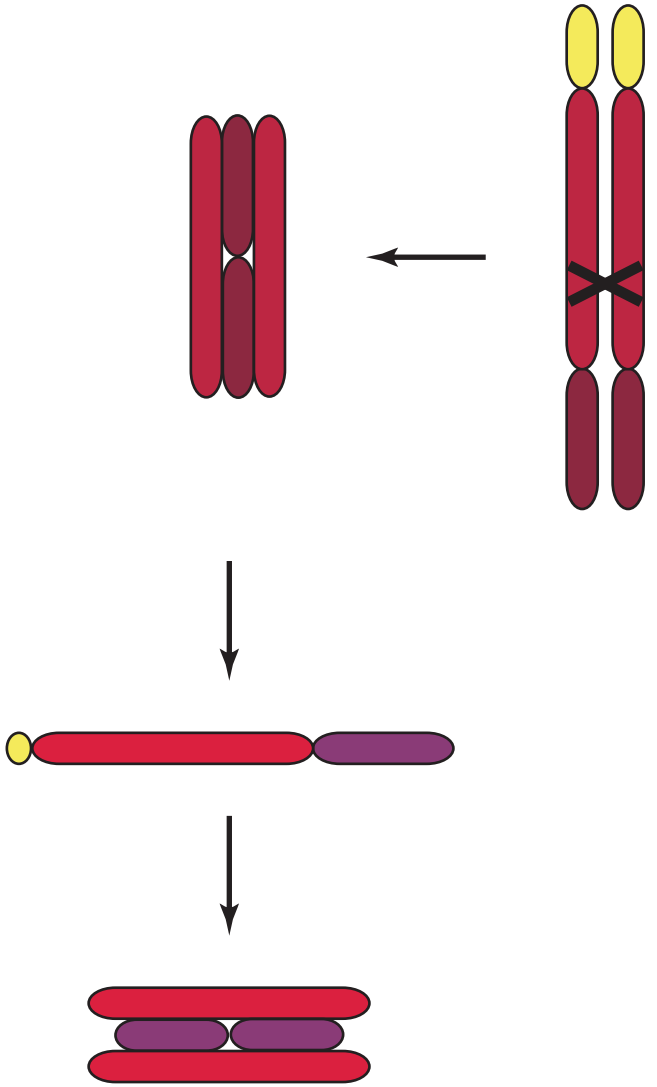


Figure 4

Stripped down to its bare essentials, two major apoptotic signaling pathways are distinguished: DR- and mitochondria-mediated apoptosis, commonly referred to as the initiator arm of apoptosis. Both pathways converge onto the effector caspases, starting with caspase-3, therefore called effector/executioner arm or common pathway. Initiator and effector pathways are shown separated by a dotted line. The minutiae of the individual initiator pathways will be discussed in the following figures.

Overview of Apoptotic Pathways

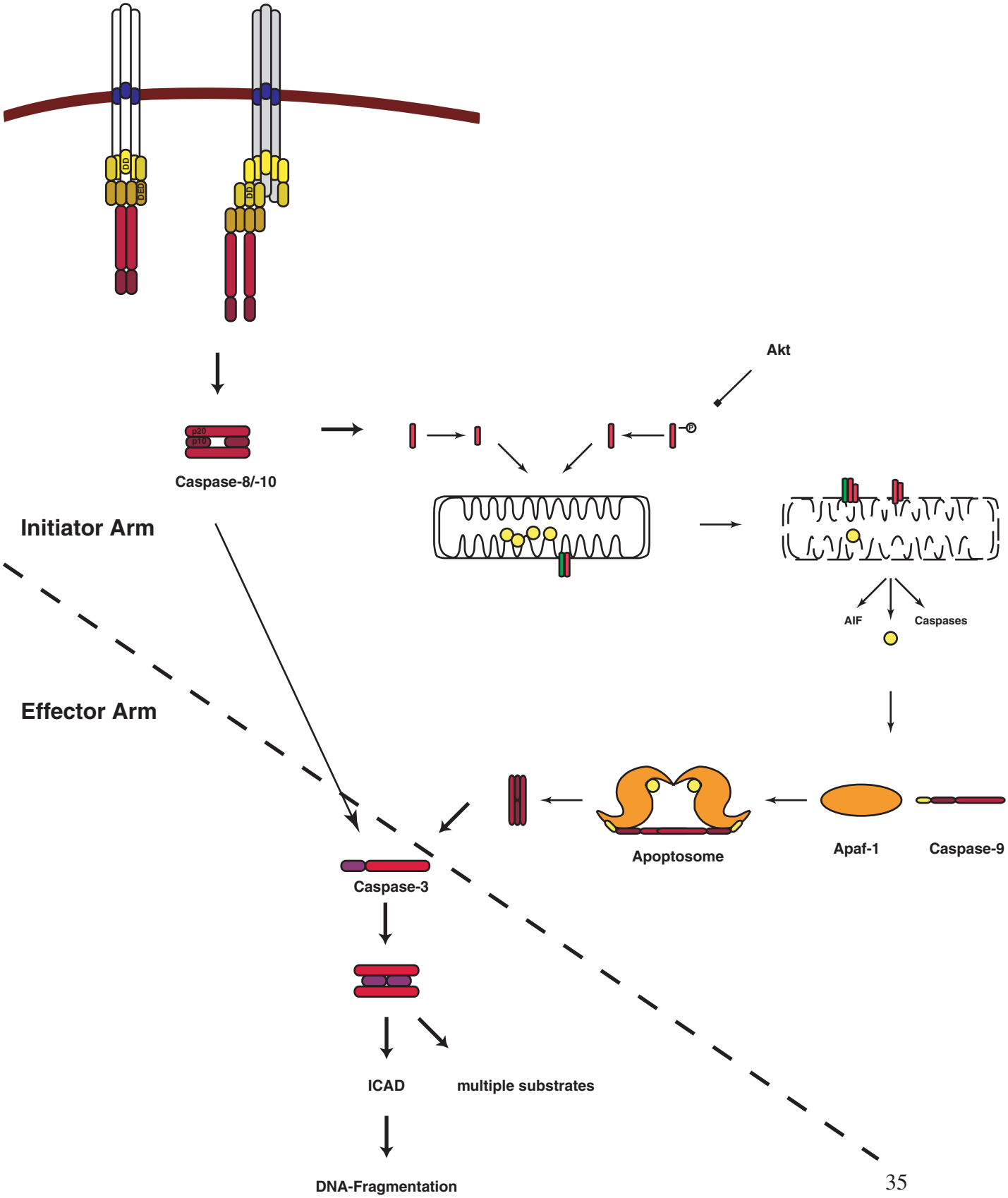


Figure 5

The mitochondrial death pathway is engaged when the balance of active pro- and anti-apoptotic Bcl-2 family members is shifted in favour of the former. This can be brought about by dephosphorylation of inactive, cytoplasmic BAD, or cleavage of BID by caspase-8. Transcriptional up- or down-regulation of Bcl-2 family members also plays an important role. As a result, anti-apoptotic Bcl-2 family members are inactivated and supernumerary pro-apoptotic family members are freed to perform pro-death activity. This leads to a breakdown of mitochondrial transmembrane potential and the opening of a large mitochondrial pore through which several effector molecules are released into the cytoplasm. Probably the most important of which is cytochrome c. Upon release, cytochrome c binds and oligomerizes Apaf-1 which then recruits pro-caspase-9 into a complex called the apoptosome. This leads to caspase-9 dimerization and subsequent processing and activation. Active caspase-9 engages the effector arm by direct cleavage of caspase-3.

The Mitochondrial Death Pathway

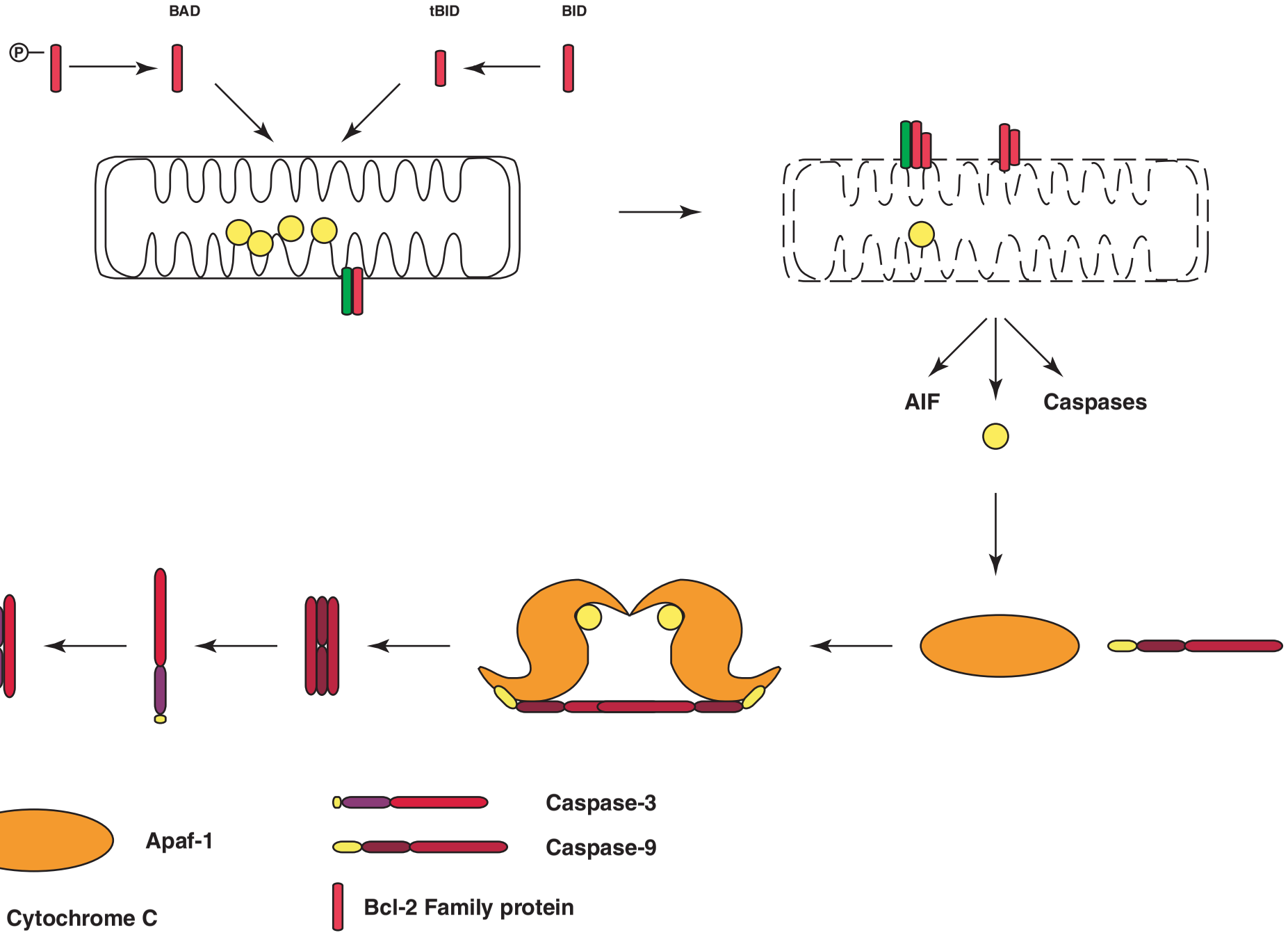


Figure 6

The Death Receptor pathway is engaged upon binding of the trimeric cytokine death-ligand to the respective receptor, which leads to trimerization of the receptor in the plane of the membrane. Receptor trimerization is followed by recruitment of adapter molecules (FADD for Fas, TRADD/FADD for TNFR) and the formation of the death-inducing signaling complex. These adapter molecules bind the initiator caspase-8 (sometimes –10), which dimerize and activate each other. The activated caspase connects to the effector arm by direct processing of caspase-3. In some cell types with low DISC activity, a mitochondrial amplification loop is required for effective apoptosis. This is achieved by caspase-8 cleavage of Bid. Activated tBid now binds and activates Bax, which leads to the execution of the mitochondrial events.

Some DR (TNFR, DR3) also engage survival pathways, through TRAF molecules, such as the NF-κB pathway that transcriptionally upregulates survival molecules.

The Death Receptor Pathway

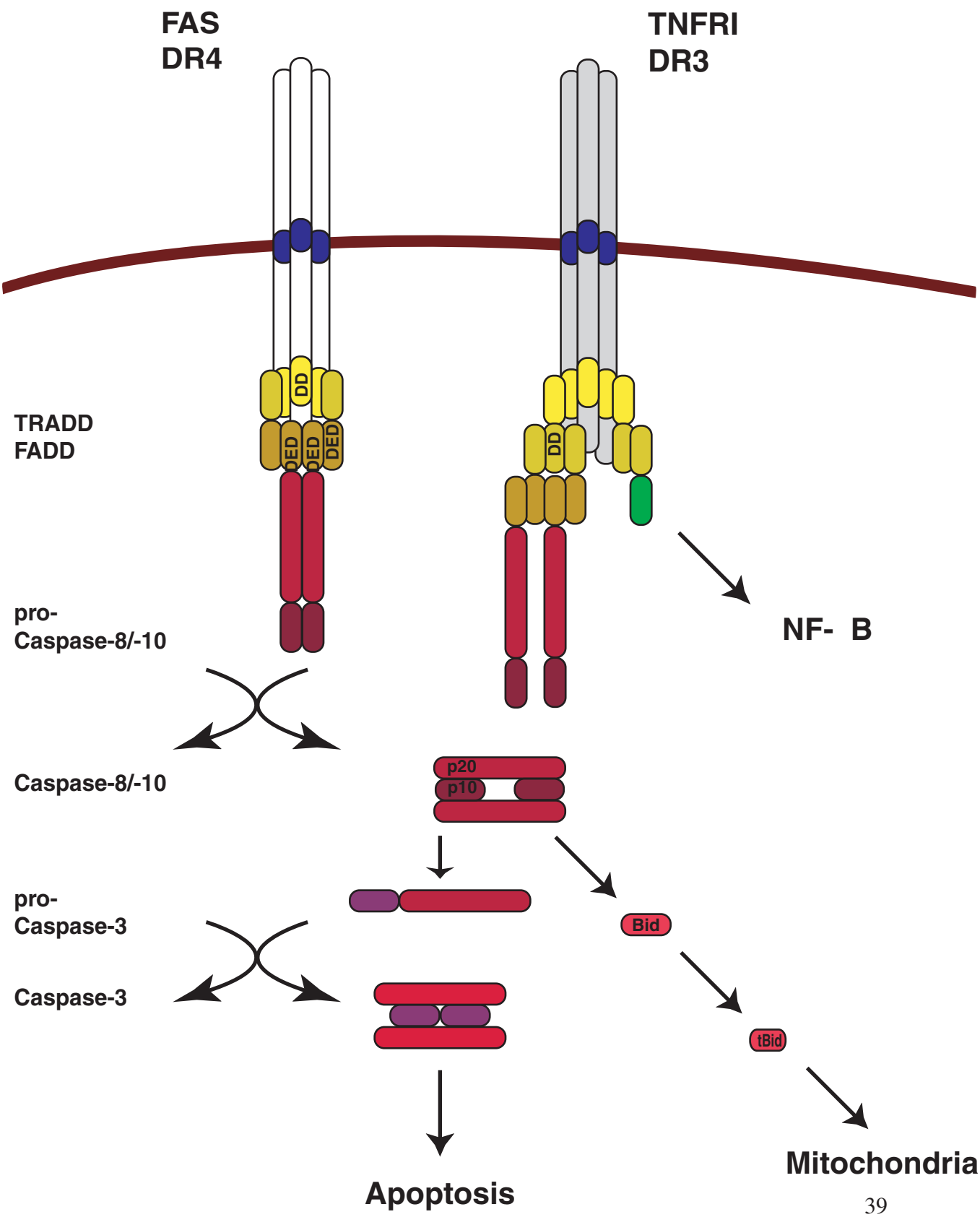


Figure 7

The *C. elegans* death machinery consists of CED-9, which binds and inactivates CED-4. CED-4 binds CED-3. Upon transcriptional upregulation of Egl-1, Egl-1 binds to CED-9 and disrupts its binding to CED-4. Released CED-4 binds and activates CED-3 which leads to cell demise.

Activation of the *C. elegans* Death Machinery

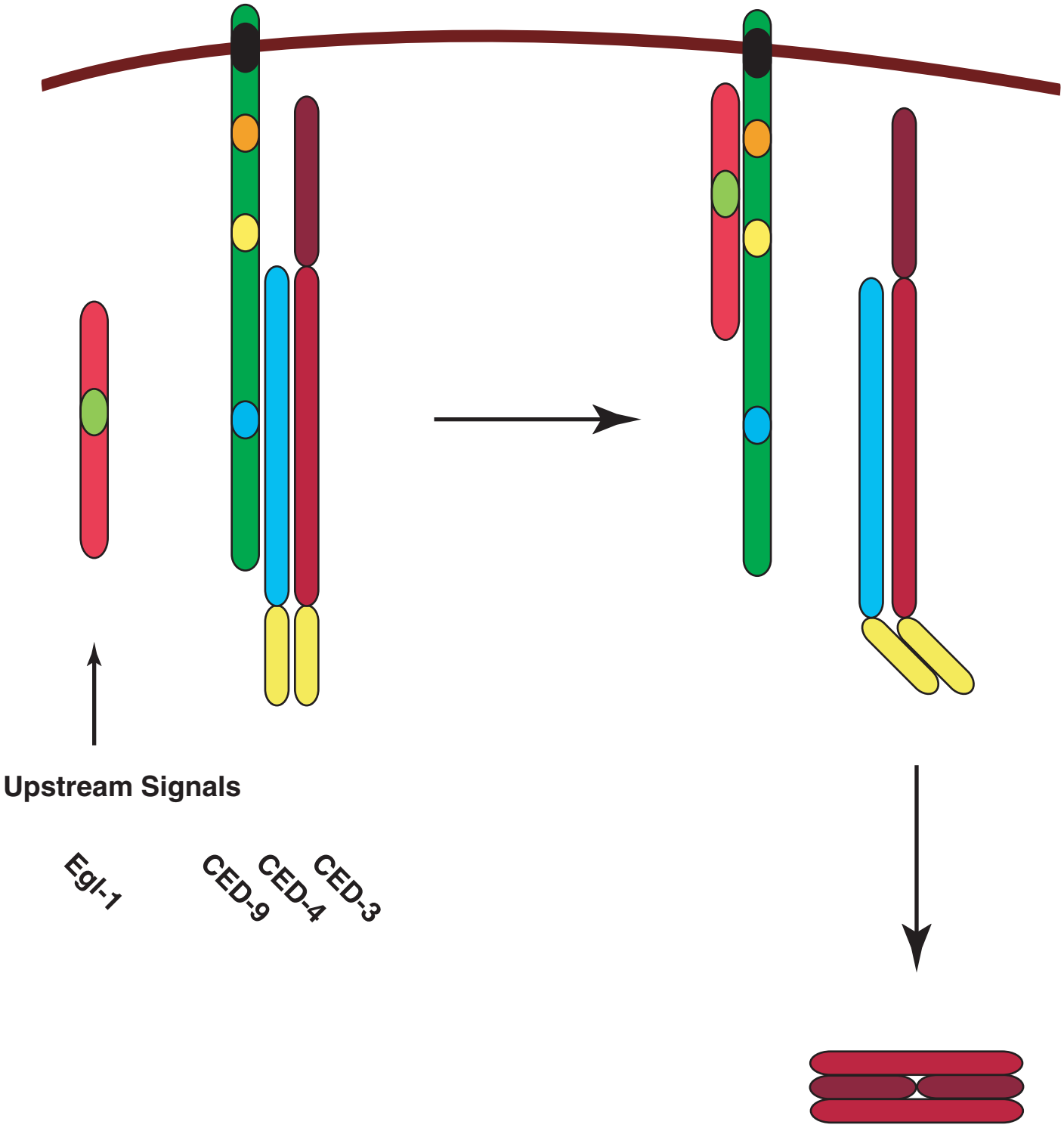
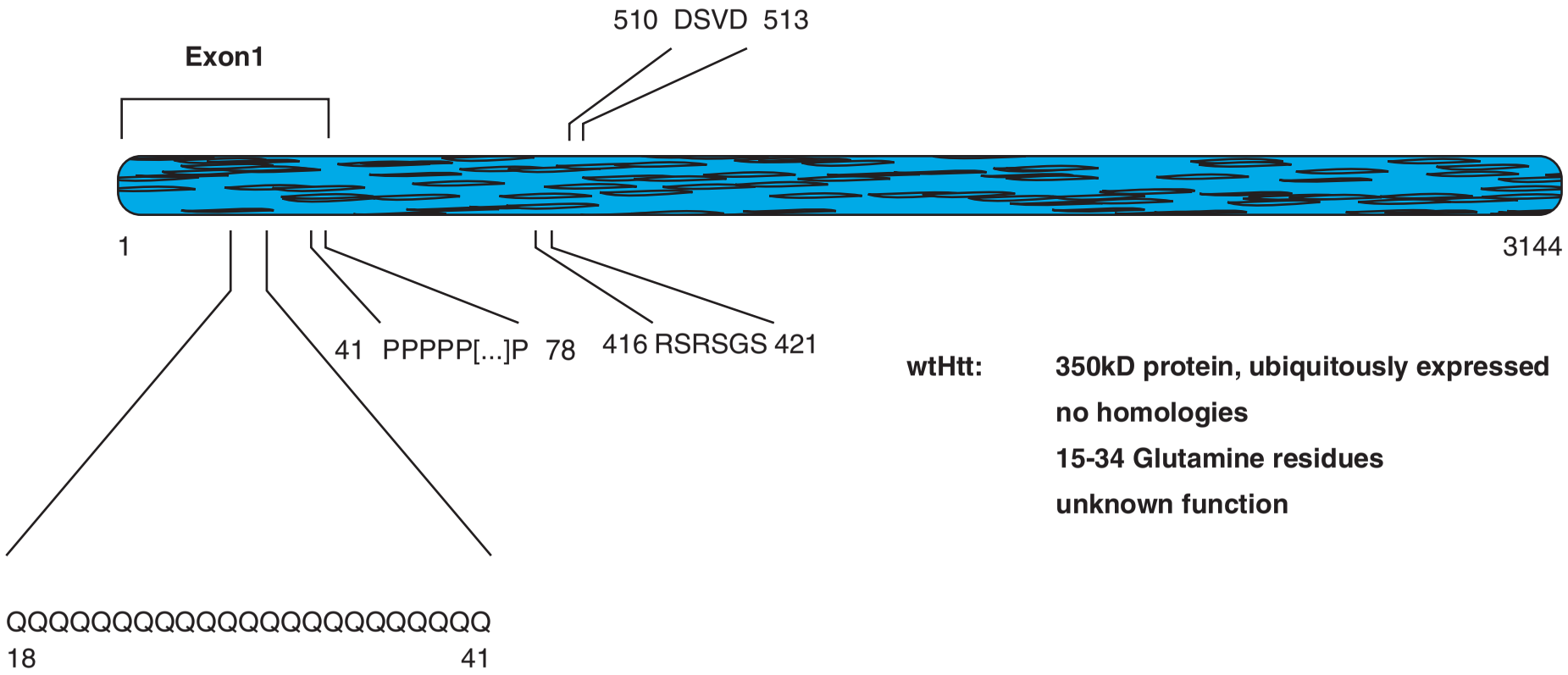


Figure 8

The Huntingtin protein is a 350kD protein that is ubiquitously expressed but shows no homologies to other proteins. It carries a stretch of glutamines in its N-terminus, which is expanded past the wildtype 15-36 residues to sometimes over 120 in Huntingtons Disease. It also contains several caspase cleavage sites, one of which at D513 is utilized by caspase-3 in HD, and a conserved phosphorylation site for Protein Kinase B/Akt.

Structure of the Human Htt Protein



wtHtt: **350kD protein, ubiquitously expressed**

no homologies

15-34 Glutamine residues

unknown function

muHtt: **37-120 Glutamine residues**

cleaved by Caspase-3 at D513

cleavage fragment forms IIN

causes HD by induction of selective cell loss of strital neurons
gain-of-function effect
pathway of neurodegeneration unknown

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