Mitochondrial dynamics in *Caenorhabditis elegans* programmed cell death

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For my parents

Love Ravi

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Statement

I have written this thesis independently without the help of others. The content of this thesis is mainly based on experiments I performed by myself, but also includes significant data which were done in close collaboration with my supervisor Dr. Barbara Conradt. Specifically, data in data tables 2 and 3, I constructed the transgenic animals and Dr. Conradt performed the analysis. Part of this work has been published recently (Jagasia et al., 2005).

Abstract

As multicellular organisms appeared in evolution, about 1.5 billion years ago, a process called programmed cell death or apoptosis evolved. Genetic studies of physiological cell death in the nematode *Caenorhabditis elegans* have elucidated a molecular model of the central cell death machinery consisting of the genes *egl-1*, *ced-9*, *-4* and *-3*. Remarkably, considering *C. elegans* evolutionarily separated from mammals millions of years ago, this pathway appears to be highly conserved from the nematode to humans. However, unlike in mammals, even though *egl-1*, *ced-4* and *-9* appear to localize to the mitochondrion *in vivo* there has been very little evidence to date that mitochondria play any role in the induction of *C. elegans* physiological cell death. This investigation demonstrates that mitochondria fragment upon induction of cell death and that this mitochondrial morphological change is not seen in *egl-1* and *ced-9* mutants but in *ced-4* and *-3* mutants. This genetic analysis suggests that mitochondrial division is an active process that occurs before caspase activation. Furthermore, the mitochondrial fragmentation event is both required and sufficient for physiological cell death in *C. elegans*. In conclusion, this work supports an important role of mitochondria in the execution of programmed cell death, suggesting that the evolutionary conservation from worm to man is stronger than previously assumed.

List of Abbreviations

A, alanine
A1, lethal antigen 1
AIF, apoptosis inducing factor
Amp, ampicillin
Apaf-1, apoptotic protease activating factor-1
ATP, adenosine 5'-triphosphate
Bad, Bcl-2 antagonist of cell death
Bak, Bcl2-antagonist killer 1
Bax, Bcl2-associated X protein
Bcl-2, B-cell lymphoma 2 protein
bcl-x, Bcl-2 related gene
Bcl-xL, Bcl-2 related protein, long isoform
Bcl-xS, Bcl-2 related protein, short isoform
Bcl-w, Bcl2-like 2
betaNAC , beta nascent polypeptide-associated complex
BH, Bcl-2 homology
BH3, Bcl-2 homology region 3
Bid, BH3-interacting domain death agonist
Bik, Bcl-2 interacting killer
Bim, Bcl-2 interacting mediator
Bok, Bcl2- related ovarian killer
Bp, base pair
Buffy, buffy
Ca^{2+}, calcium
cAMP, cyclic adenosine 3',5'-monophosphate
caspase, apoptosis-related cysteine protease
Ced, cell death abnormality
C. elegans, *Caenorhabditis elegans*
cps, CED-3 protease suppressor
csp, caspase
C-terminus, carboxy terminus
Dark, darkener of white-eosin
ddH_2O, double distilled H_2O
Debcl, death executioner Bcl-2 homologue
DIABLO, Direct IAP-binding protein with low pI
DIC, differential interference contrast
dn, dominant negative
DNA, deoxyribonucleic acid
Dnm-1p, dynamin protein 1
dpy, dumpy, shorter than wild-type
Drob1, death related ovarian Bcl-2 1
drp, dynamin related protein

D. melanogaster, drosophila melanogaster
dsDNA, double stranded DNA
dsRNA, double stranded RNA
E, glutamate
E. coli, Escherichia coli
eat, eating: abnormal pharyngeal pumping
egl, egg laying defective
EndoG, endonuclease G
erp, endophilin related protein
et al., Et alii
EtOH, ethanol
F1, first generation
F2, second generation
Fas, Tumor Necrosis Factor receptor superfamily, member 6
Fig., figure
fis, S. cerevisiae FIS1-related
Fis1p, fission 1
Fzo1p, fuzzy onions;
g, gram
G, glycine
GDP, guanosine diphosphate
GED, GTPase effector domain
gf, gain of function
GFP, green fluorescent protein
GTP, guanosine triphosphate
GTPase, guanosine triphosphatase
H_2O, water
hfis-1, human fission 1
hr, hour
Htra2, HTRA, E. Coil, HOMOLOG OF 2
IAP, inhibitor of apoptosis
icd, inhibitor of cell death
i.e., Id est
IMS, inter membrane space
in vitro, within glass
in vivo, within the living
IPTG, isopropyl β-D-thiogalactoside
K, lysine
kDa, kilodalton
KH_2PO_4, potassium phosphate
L1, larvae stage 1
L4 larvae stage 4
LB, Luria Bertain
LCS, Leica confocal software
lf, loss of function
M9, M9 salt medium

M4, pharyngeal muscle cell 4
MAP, mitogen activated protein
Mcl-1, myeloid cell leukemia 1
Mdm33p, Mitochondrial distribution and morphology 33
Mdv-1p, mitochondrial division 1
l-Mgm-1, long mitochondrial genome maintenance form
s-Mgm-1, short mitochondrial genome maintenance form
$MgSO_4$, magnesium sulfate
µm, mikrometer
min, minutes
Mdv-1p, mitochondrial division 1
Mfn, mitofusin
mitogfp, mitochondrial targeted GFP
mtDNA, mitochondria DNA
myo-3, myosin heavy chain structural gene *3*
NaCl, sodium chloride
NGF, neuronal growth factor
NGM, nematode growth medium
N terminus, amino terminus
OPA1, optic atrophy 1 gene
P, phosphate
$P_{egl\text{-}1,}$ *egl-1* promoter
$P_{hs,}$ heat shock promoter
P_{myo3}, *myo3* promoter
PCD, programmed cell death
pH, $-\log_{10}[H^+]$
pI, isoelectric point
Puma, p53-upregulated modulator of apoptosis
RNA, ribonucleic acid
RNAi, RNA interference
Rol, roler
rrf , RNA-dependent RNA polymerase Family
s, seconds
S, Svedberg
S. cerevisiae, Saccharomyces cerevis
Smac, second mitochondria-derived activator of caspase
Src, sarcoma
TNF, tumor necrosis factor
TMRE, tetramethylrhodamine ethyl ester perchlorate
unc, uncoordinated
wah, worm AIF homolog
WD, Tryptophan-Aspartate
Z-VAD-FMK, N-Benzyloxycarbonyl-valinine, alanine, aspartate-fluoromethyl ketone

1 Introduction

First, a brief introduction into the study of programmed cell death (PCD) will be given, it will then be elaborated on why *C. elegans* is synonymous with this process. Then a critical discussion into the evolutionary conservation and diversity of this process in other animals, particularly in mammals, which diverged from *C. elegans* hundreds of millions of years ago, will be offered. Then a detailed description of the molecular mechanisms of PCD in *C. elegans* will be presented and critically discussed what is still unknown and unclear, particularly with respect to the role of the mitochondrion. This discussion will reveal why this investigation was initiated. Then an introduction into an emerging and important process in biology, mitochondrial dynamics will be given, summarizing what is known about its molecular components and its function and why it is important to study this process in *C. elegans*. Finally, a description into two processes of mitochondrial dynamics in more detail will be outlined, fission and fusion, addressing what are the components that regulate these processes and why they may be important in PCD.

1.1 Programmed cell death

Programmed cell death (PCD) or apoptosis is an evolutionarily conserved program of cell suicide. This cell termination pathway evolved to eradicate excess and potentially dangerous cells in most if not at all eukaryotic organisms. Unwanted cells are generated

throughout an organism's plight, both in development and later in adulthood. When this genetically encoded pathway falters it can have detrimental consequences to specific organism. Indeed, in humans, cases of hypo-apoptosis lead to the formation of various cancers (Sellers and Fisher, 1999; Fulda and Debatin, 2004). Conversely hyper-apoptosis can be involved in degenerative pathologies such as multiple sclerosis and Alzheimer's disease (Kusiak et al., 1996; Zipp, 2000; Zipp et al., 2002; Cribbs et al., 2004).

It is thought that PCD evolved in multicellular organisms to balance the complexity of shape formation, the specific process of forming distinct structures from proliferating cells (Brill et al., 1999). This hypothesis assumes that it was easier for an organism to evolve processes to kill cells rather than to specifically produce the specific amount of cells required. The evolutionary pressure to drive the formation of cell suicide was likely to block another form of cell death from proceeding, namely necrosis, which can be viewed as the explosion of cells. Necrotic spillage of subcellular components of the dying cells is toxic to their neighbors (Huettenbrenner et al., 2003). This can be seen in the human brain during ischemia (Scarabelli and Gottlieb, 2004). A specific region of tissue is initially affected and dies by necrosis, the penumbra (Martin et al., 1998). The region of necrotic death then rapidly expands due to the cellular toxicity of cell explosion (Scarabelli and Gottlieb, 2004). Thus, it is likely that organisms' evolved mechanisms termed apoptosis or cell suicide to control the potentially dangerous process of cell death.

It is thought that with the emergence of multicellularity, apoptosis appeared. It can therefore be assumed that the fundamentals of PCD would be preserved in a variety of

organisms, for example from worm to human. Indeed, on first glance this appears to be the case, as the main features of PCD are conserved between nematodes such as *C. elegans*, insects such as *Drosophila melanogaster* and mammals such as *Homo sapiens*. This conservation is seen at the level of both the genes encoding the basic cell death machinery (discussed in subsequent sections) and the morphological and biochemical features accompanying apoptosis. The morphological characteristics can be viewed as cell implosion and include fragmentation of the cell with an initial retention of organelle structure, chromatin condensation, and cleavage of DNA into smaller fragments and the subsequent rapid engulfment of the suicidal cell by phagocytes (Kerr et al., 1972; Ellis et al., 1991; Hacker, 2000). All these morphological features effectively allow the cell to disappear without a trace.

One interesting point is that although we assume PCD arose with multicellularity and thought not to serve a selective advantage in unicellular organisms, several findings have indicated that a similar process operates in single-celled eukaryotes and prokaryotes. PCD has now been described in species of unicellular eukaryotes which phylogentically diverged at least 2 billion years ago, such as *Saccharomyces cerevisiae* (Madeo et al., 1997) and *Schizosaccharomyces pombe* (Jurgensmeier et al., 1997), *Trypanosoma brucei* (Debrabant and Nakhasi, 2003), *Leishmania* (Zangger et al., 2002) and the slime mold *Dictyostelium discoideum amazonensis* (Cornillon et al., 1994). Thus the rudimentary roots of cell death may have evolved earlier than previously assumed. A plausible hypothesis is that apoptosis evolved to combat the selective pressures of limited nutritional supply of an organism's specific niche (Herker et al., 2004). For these reasons,

when we consider the emergence of cell death we must remember that a primitive form existed prior to the appearance of the multicellular organism *C. elegans*.

The study of cell death has been comprehensively studied in a variety of organisms, each with specific advantages and disadvantages. The present work has been done in the model organism *C. elegans,* the simplest multicellular organism for the genetic analysis of programmed cell death. In the next sections, the field of *C. elegans* programmed cell death will be introduced continuously emphasizing and comparing the similarities and differences of apoptosis in other organisms, particularly mammals, and briefly in the insect *D. melanogaster* and the unicellular eukaryote *S. cerevisiae*, since these organisms have as well been extensively studied.

1.2 Introduction to programmed cell death in *C. elegans*

The paradigm initially observed by Horvitz and coworkers, which at the time was provocative, but which has now changed the course of many fields biology, was that programmed cell death has a genetic component (Horvitz et al., 1983; Ellis and Horvitz, 1986; Avery and Horvitz, 1987). These observations were made in the nematode *C. elegans* and subsequently extended to mammalian systems (Vaux et al., 1988), insects (Steller et al., 1994; White et al., 1994), and other metazoan (Cikala et al., 1999), and finally *S. cerevisiae* (Madeo et al., 1997). In the course of *C. elegans* development, 131 of the 1090 somatic cells formed die by programmed cell death (Sulston and Horvitz, 1977; Sulston et al., 1983). This is mediated by a genetic pathway since mutations in first

two, and later four genes were reported to prevent the loss of these 131 cell death events (Ellis and Horvitz, 1986; Hengartner et al., 1992; Conradt and Horvitz, 1998) . Since mutations in these four genes block almost all of the somatic cell death events, they comprise the core cell death pathway. The mutations were loss-of-function mutations of the genes *ced-3 (ced,* cell death abnormality*), ced-4* and *egl-1 (egl,* egg laying defective), and a gain-of function mutation of *ced-9.*

Obviously these four core genes cannot co-ordinate the specificity, killing and removal of all 131 individual cells destined to die. In fact, genetic screens have led to the identification of over 15 different genes involved in various aspects of physiological cell death in *C. elegans* (Xue et al., 2002; Horvitz, 2003). Researchers sometimes divide programmed cell death into several "sequential and distinct phases" defined by the various mutant phenotypes observed (Wu and Xue, 2003). 1) Decision phase, consisting of the specific factors that confer the cell fate, or the decision to die. Animals with mutations of genes specific to this phase display a phenotype where some but not all somatic cell death events are blocked. 2) The execution phase, consisting of the process by which all somatic cells are executed to die. Animals with mutations in genes specific to this phase display a phenotype where all somatic cell death is blocked. 3) The phagocytic phase, consisting of the recognition and engulfment of the dying cells by neighboring cells. Animals with mutations of genes required for this phase display a phenotype where the dead cells are not removed and the morphological remnants persist as cell corpses. However, recent reports demonstrate that phagocytosis can actively promote the execution phase (Hoeppner et al., 2001; Reddien et al., 2001). Thus, a re-

evaluation of how independent and sequential the phases of programmed cell death is required, as they are not as mutually distinct as initially thought. As well, in the male, neighboring cells are directly required for some cell death events to proceed (Sulston et al., 1980). 4) Final step, the remnants of the engulfed cells in the neighboring cell are degraded. Animals, with mutations of genes specific to this phase display phenotypes where the effector targets of the active caspase are blocked, such as DNA degradation. This phase is also not an independent entity, but requires the activity of both the dying and phagocytic cell and proceeds concomitantly with engulfment (Wu et al., 2000). Furthermore it has been reported that the degradation of DNA can in some cases block cell death from proceeding, further reinstating that the phases are not separate events (Parrish et al., 2001; Wang et al., 2002). Interestingly, microscopists have reported that the engulfment of cells destined to die can proceed even before the completion of cytokinesis of the mitosis that generated them (Robertson and Thomson, 1982). This work focuses specifically on the execution phase of cell death. Thus this process and its subsequent players will be considered in greater lengths.

To summarize a couple decades of extensive, predominantly, genetic research by Horvitz and coworkers and supporting biochemical studies from other laboratories (Fig. 1) (Chinnaiyan et al., 1997; Metzstein et al., 1998; Yang et al., 1998; del Peso et al., 2000; Xue et al., 2002; Horvitz, 2003; Wu and Xue, 2003): in cells destined to survive, CED-4 interacts with CED-9 at the mitochondria, while the *egl-1* gene is not transcribed and CED-3 remains inactive likely in the cytosol. In cells destined to die, *egl-1* transcription is activated through factors composing the decision phase. The EGL-1 protein likely translocates to the mitochondria where it interferes with the CED-9/CED-4 interaction.

This triggers the release of CED-4 into the cytosol where it translocates to the perinuclear membrane subsequently activating CED-3, the effector of death. This scenario can be viewed as a simple genetic model (Fig.1a). CED-3 and CED-4 are pro-apoptotic, CED-9, the anti-apoptotic molecule, inhibits CED-4, and the pro-apoptotic EGL-1 initiates death through inhibition of the anti-apoptotic CED-9.

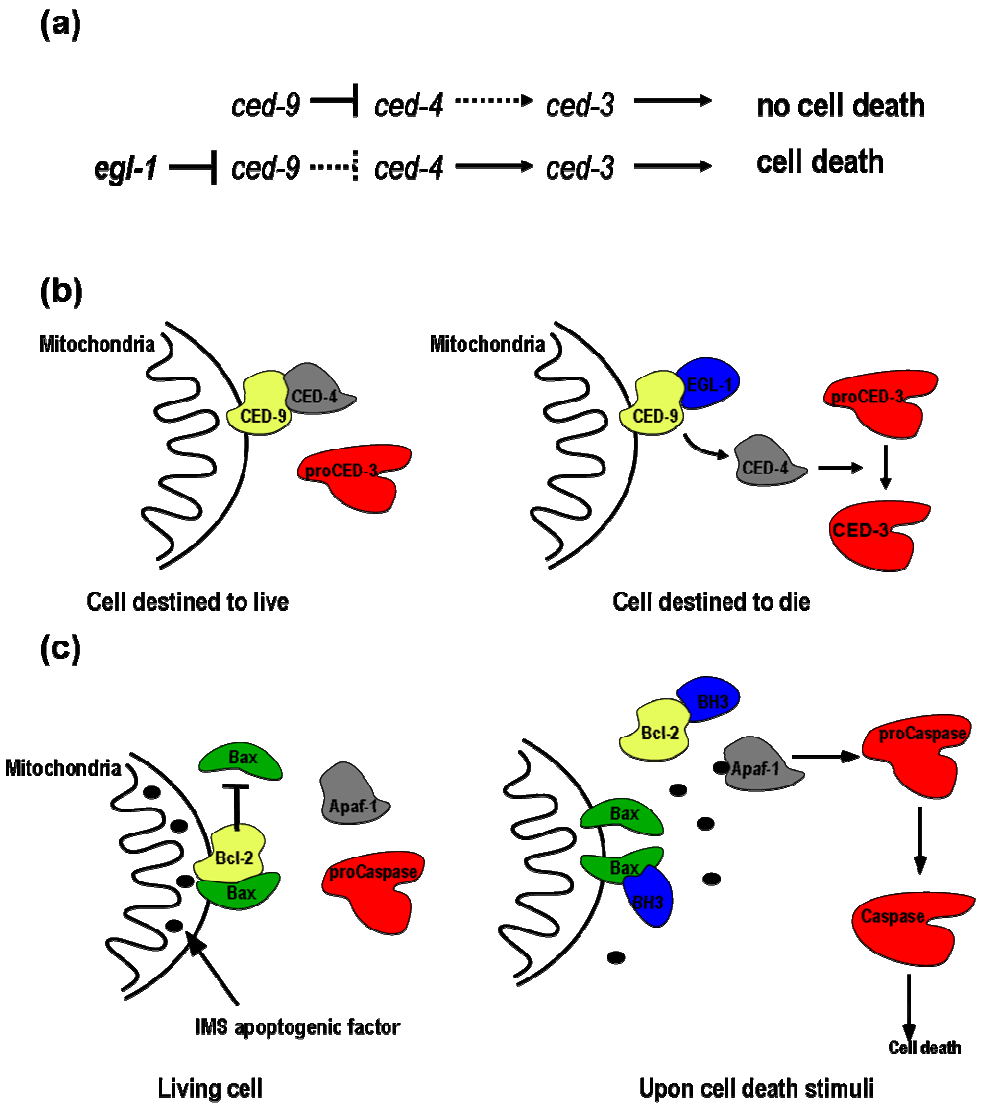

Figure 1. Overview of programmed cell death in *C. elegans* and mammals.
(a) Genetic model of the cell death pathway in the 959 cells destined to live (top panel) and the 131 cells destined to die (bottom panel). Solid arrows and T-bars indicate activation and suppression respectively, and dotted arrows and T-bars indicate suppression and activation respectively that did not occur because the cell either lived or died. (b) Molecular model corresponding to the above genetic pathway in *C. elegans*. (c) Molecular model of cell death in mammals. *C. elegans* homologues are depicted in the same color as in (b). Abbreviations: CED, cell death abnormality; EGL, egg laying defective; IMS, inter membrane space, Bcl-2, B-cell lymphoma 2 protein; BH3, Bcl-2 homology region 3, Bax, BCL2-associated X protein; Apaf-1, apoptotic protease activating factor-1 (Adapted from: Metzstein et al., 1998; Gross et al., 1999).

Depicted in Fig. 1c, all 4 of these *C. elegans* proteins have homologues in mammals and likely in insects, which perform similar functions in apoptosis regulation. The initiator of *C. elegans* cell death, EGL-1 is a BH3-only protein, which are cell death activators that include the mammalian proteins Bik, Bid, Puma and Bad (Conradt and Horvitz, 1998; Huang and Strasser, 2000) and, as of yet, but not likely for long, no homologue *D. melanogaster* exists. The likely reason a BH3-only protein in insects has yet to be discovered is that they are difficult to identify due to low sequence conservation and small region of homology (about 9 amino acids). The inhibitor of cell death, CED-9, encodes a protein of the Bcl-2 protein family including mammalian members Bcl-2 (Hengartner et al., 1992; Hengartner and Horvitz, 1994a), Bcl-xL, Bcl-w, Mcl-1, A1 (Huang and Strasser, 2000) and Buffy in *D. melanogaster* (Quinn et al., 2003; Quinn and Richardson, 2004) which also all confer anti-apoptotic functions. The pro-apoptotic protein CED-4 has only one homologue in both mammals, Apaf-1 (Yuan and Horvitz, 1992; Zou et al., 1997) and *D. melanogaster*, Dark (Rodriguez et al., 1999) . The downstream effector in this execution pathway, CED-3, is a protein that is a member of a family of death inducing proteases called caspases (Yuan and Horvitz, 1990; Yuan et al., 1993) which cleave substrates after specific aspartate residues and mediate the cellular degradation and morphological changes seen during apoptosis (Vaux and Strasser, 1996; Riedl and Shi, 2004). Except for CED-4, there exist significantly more homologues of the single core component from *C. elegans* in mammals. This raises the question whether the complexity of PCD in mammals could simply be explained by the increased diversity of

the same components for the diverse amounts of specific cell death scenarios. The answer to this question is likely, no.

To summarize extensive work from the mammalian apoptosis field (Fig.1c) (Gross et al., 1999; von Ahsen et al., 2000; Marsden and Strasser, 2003) :In living cells, BH3-only proteins (EGL-1 homologues) are sequestered inactive and the Bcl-2 protein (CED-9 homologue) directly inhibits the Bax-like proteins, such as Bax and Bak, at the mitochondria, or Bax is sequestered inactive in the cytosol. Apaf-1 (CED-4 homologue) and caspase (CED-3 homologue) are inactive in the cytosol. In cells induced to die, the BH3-only proteins become active, and then might directly activate Bax or de-repress Bax through inhibition of Bcl-2, leading to the release of apoptogenic factors from the inter membrane space. One of these factors, Cytochrome *c*, upon release, can bind and activate Apaf-1, which in turn can induce the activation of caspases (Zou et al., 1997).

1.3 Similarity and diversity in programmed cell death between *C. elegans* and mammals

To reinstate what was mentioned above, most of the 15 different genes of the *C . elegans* cell-death pathway with integral roles in cell death have mammalian counterparts, reconfirming that the process of programmed cell death in *C. elegans* is similar to that in humans. How similar is the execution of cell death? Four major break points between the two organisms is presented below, (1) Bax-like proteins, (2) CED-4/Apaf-1, (3) release

of Cytochrome *c* from the inter membrane space, (4) release of other apoptogenic molecules from the inter membrane space.

1.3.1 Bax-like proteins

A comparison of Fig.1b and 1c illustrates that in mammals there is an extra player depicted in green, the pro-apoptotic molecules, Bax-like proteins such as Bax. These Bax-like proteins include mammalian proteins Bax, Bak, Bok, and Bcl-x_s (a splice variant of the *bcl-x* gene) (Marsden and Strasser, 2003) and *D. melanogaster,* Debcl and Drob-1 (Colussi et al., 2000; Igaki et al., 2000). Bax functions to promote cell death at a step distinct from the BH3-only proteins (in *C. elegans* EGL-1). In mammalian cells, the multi-domain Bax is somehow responsible for increasing the permeability of the outer mitochondrial membrane, maybe by forming a channel, which permits the release of factors such as Cytochrome *c* from the inter membrane space. It is possible that this step is not required in *C. elegans* programmed cell death. However it can not be ruled out that CED-9, the inhibitor of cell death, which contains the BH regions of Bax and appears to be structurally very similar, could confer this pro-apoptotic function. It could be that binding of EGL-1 to the mitochondrial CED-9/CED-4 complex and/or release of CED-4 from it leads to CED-9 adopting a killing function. EGL-1 would then have a similar function to two mammalian BH3-only proteins, Bid and Bim, which can directly activate Bax and potently induce Cytochrome *c* release from mitochondria in cultured cells (Kuwana et al., 2005). If this scenario were valid, either CED-9 would confer a killing and/or CED-4 a survival function through inhibition of CED-9. Indeed, both proteins

have been shown to adopt dual functions in programmed cell death (discussed below 1.4.2) (Hengartner and Horvitz, 1994b; Shaham and Horvitz, 1996b).

1.3.2 CED-4

A clear distinction between mammalian and *C. elegans* cell death comes from CED-4. In *C. elegans*, CED-4 is inhibited from activating the effector caspase, CED-3, by being sequestered at the mitochondrion through CED-9 (Wu et al., 1997; Chen et al., 2000). In mammals and *D. melanogaster* on first glance this is not the case. Apaf-1 and Dark, CED-4 homologues respectively, seem to be localized in the cytosol (Zou et al., 1997; Rodriguez et al., 1999). Only through the release of an apoptogenic factor from the mitochondrion does Apaf-1 become active (Zou et al., 1997; Rodriguez et al., 1999).

Unlike CED-4, which binds to CED-9, Apaf-1 does not appear to bind to Bcl-2 (or its homologs). Furthermore, both Apaf-1 and Dark contain 12 or 13 C-terminal WD40 repeats, which have been shown necessary for interactions with the mitochondrial protein Cytochrome *c* (Zou et al., 1997; Rodriguez et al., 1999). Upon release of Cytochrome *c* from the mitochondria, it activates Apaf-1's apoptotic function through direct interaction with these WD40 repeats. Since CED-4 lacks these WD40 repeats there is no evidence for a role of Cytochrome *c* in CED-4 activation and programmed cell death in *C. elegans*. However, even though CED-4 and Apaf-1 are structurally similar there is no proof that these molecules are functionally homologous. It would be interesting to attempt to rescue a *ced-4(lf)* with mammalian Apaf-1; if this was successful this would provide direct evidence that these molecules are functionally homologous. Interestingly, Dark, similar

to its mammalian counterpart is required for many apoptotic responses in the fly, and contains the carboxy-terminal WD40 repeat domain but in striking contrast to Apaf-1 does not appear to require Cytochrome *c* for activation (Kanuka et al., 1999; Rodriguez et al., 1999; Zhou et al., 1999). These studies in *Drosophila* raise the question of whether Cytochrome *c* is the only activator of Apaf-1.

Interestingly, there is reason to believe that both CED-4 and Apaf-1 mediated cell death could require yet unidentified components, leading to the possibility that the process of cell death through the release of Cytochrome *c* may be more comparable from worm to human.1) Mammalian Bcl-2 can rescue *C. elegans ced-9(lf)* mutants from ectopic cell death (Hengartner and Horvitz, 1994a), suggesting that they are true functional homologues. Since the subcellular localization of CED-4 correlates with the cell-death status of a cell, in cells committed to live, mitochondrial, and in cells committed to die, perinuclear (Chen et al., 2000), it has been proposed that CED-9's anti-apoptotic functions is to sequester CED-4 at the mitochondria (Chen et al., 2000). If this were the case, in order for mammalian Bcl-2 to rescue *ced-9(lf)* mutants it must be redirecting CED-4 mislocalization which is presumably causing the ectopic cell death in the mutant animals. This hypothesis is plausible since it was shown that CED-4 interacts with mammalian Bcl-xl, a mammalian CED-9 homolog in mammalian cells (Wu et al., 1997). This raises the possibility that in the mammalian system, Bcl-2 may also inhibit and sequester the true CED-4 functional homologue at the mitochondria. 2) Most organs of Apaf-1-deficient mice develop normally and in some cases Apaf-1 null mice survived until adulthood with no apparent defects except for male sterility (Yoshida et al., 1998; Yoshida, 2003). In striking contrast, disruption of *ced-4*, blocks all physiological

programmed cell death events in *C. elegans*. Since presently, Apaf-1 is the only gene in the mammalian genome known to show homology to *ced-4* and it appears that many mammalian developmental cell death events require caspase-3 activation (Kuida et al., 1996), presumably apoptosis proceeds in an Apaf-1 independent manner. It would then be likely that another CED-4 functional homologue exists, involved in Apaf-1 independant caspase-3 activation. 3) Even though Apaf-1 is the sole recognized mammalian CED-4 homolog, the two molecules are structurally dissimilar. Both Apaf-1 and CED-4 contain a caspase recruitment domains and nucleotide binding domain, however the rest of the proteins are quite dissimilar with CED-4 completely lacking the carboxy-terminal WD-repeat domain. Could a yet unidentified *C. elegans* protein mediate binding of Cytochrome *C* and subsequent cell death stimulation?

1.3.3 Release of Cytochrome *c* from the inter membrane space

The biggest difference from worms to humans in cell death is the fact that in the early stages of most cell death pathways in mammals the mitochondrion plays a very active role in the induction of cell death (see Fig. 1c). The so called mitochondrial pathway promotes the induction of death through the release of a plethora of molecules from the inter membrane space of the mitochondrion (Fig. 1c) that proceeds concomitantly with the initiation of death and can directly cause and or enhance the cell death cascade (Breckenridge and Xue, 2004). As of yet, very little evidence exist that mitochondrial housed proteins do or do not play a role in *C. elegans* physiological cell death. This paucity of knowledge can be explained by two reasons, namely research approaches and

the nature of the worm. 1) Most *C. elegans* researchers attempt to elucidate function through forward or reverse genetics. For this reason, identification of novel cell death components will be limited to the way the screen is performed. Since presently no screens for essential cell death components were reported, screens to date have been favoring the identification of cell death components that are non-essential. Since it appears that most if not all of these mitochondrial proteins that get released during cell death in mammals have functions in normal mitochondrial physiology, they will likely be essential, as mitochondria are essential for life. For this reason, it is not possible to test a direct role of mitochondria in cell death. Thus apoptosis researchers can not simply assume that mitochondria are not involved in physiological cell death in *C. elegans*. 2) Programmed cell death occurs predominantly in the embryo, which is protected by a chitin shell. This egg shell makes it very hard to manipulate the embryo and to perform biochemistry. In mammalian systems, one can simply watch cell death in a tissue culture dish, easily isolate protein lysates, and obtain kinetics on the release of apoptogenic molecules from the mitochondria. These experimental advantages were essential to assess the contribution of the mitochondria upon exposure to cell death stimuli. As of yet no novel techniques have yet been established, to better understand the cell death process in *C. elegans* on the level of the mitochondria. One possibility would require reconstitution of cell death in *C. elegans in vitro* with purified mitochondria to establish a direct relationship between the release of *C. elegans* IMS proteins such as Cytochrome *c* upon cell death activation. Or, and what formed the basis of the work described below, *in vivo* monitoring of physiological cell death on the level of the mitochondrion.

Cytochrome *c* was the initial and most famous mitochondrial inter membrane space protein identified that is unleashed and required during cell death in mammals (Liu et al., 1996; Yang et al., 1997). How Cytochrome *c* transverses the outer lipid bilayer of the mitochondria to reach the cytosol is still the matter of constantly revolving debate (Scorrano and Korsmeyer, 2003). What is obvious is that the mechanism is regulated by proteins of the Bcl-2 family, many of which can bind directly to the outer mitochondrial membrane (Huang and Strasser, 2000; Marsden and Strasser, 2003; Scorrano and Korsmeyer, 2003). Upon release from the organelle, Cytochrome *c* binds to Apaf-1 (CED-4 homologue) through a WD40 repeat domain, which is not present in CED-4 (see section 1.3.2). This Apaf-1/Cytochrome *c* complex then proceeds to activate caspases (Fig. 1c). As mentioned, CED-4 does not likely interact with Cytochrome *c*. However, this does not mean that another protein, maybe even in complex with CED-4, mediates CED-3 activation and subsequent death. This speculation is not too far since over-expressing active CED-4 does not as effectively induce an increase of cell death in *C. elegans* as compared to overexpressing EGL-1, the upstream initiator of the cell death cascade (Ravi Jagasia, Unpublished observation). Could EGL-1, like in the mammalian system, upon translation and translocation to the mitochondria cause the release of factors, such as Cytochrome *c*, that acts concomitantly with CED-4 release? Of interest, MAC-1, an essential *C. elegans* protein, was shown to interact and inhibit CED-4 *in vivo* (Wu et al., 1999). Could the release of an Cytochrome *c* be required to inhibit this inhibitory interaction?

1.3.4 Release of other apoptogenic molecules

As mentioned above, it has not been directly tested whether Cytochrome *c* is released from the mitochondria upon cell death induction in *C. elegans* (see section 1.3.3). In mammalian cells upon induction of cell death, a plethora of other inter membrane space proteins are released, which then perform various apoptotic functions. These molecules include Cytochrome *c*, Smac/DIABLO, Omi/Htra2, endonuclease G (EndoG) and apoptosis-inducing factor (AIF) (Breckenridge and Xue, 2004). Recently it was reported that two proteins are released from the inter membrane space during *C. elegans* cell death, *wah-1* (worm AIF homolog) and *cps-6* (CED-3 protease suppressor) (Parrish et al., 2001). This suggests that Cytochrome *c* could be released as well. Unfortunately, this work can only be taken with a grain of salt. *cps-6* encodes the homologue of human mitochondrial EndoG (Parrish et al., 2001), which is involved in the fragmentation of DNA during cell death. Reduction of *cps-6* activity caused a block in DNA degradation and a delay and slight block in cell death in *C. elegans*. These events occurred downstream of the caspase CED-3. The authors claimed that upon induction of death, CPS-6 is released from the inter membrane space to degrade DNA in the nucleus. However this was not demonstrated directly, but only concluded indirectly through evidence obtained using a truncated CPS-6 fusion protein, whose mitochondrial targeting sequence is absent. It can not be ruled out that *cps-6* has a dual localization in all cells, both mitochondrial and nuclear, a property displayed by a variety of proteins (Sass et al., 2001; Strobel et al., 2002; Sass et al., 2003). In this case it would not need to be released from the inter membrane space. Recently, it was reported that the *C. elegans* homolog of AIF, *wah-1,* might be implicated in worm programmed cell death (Wang et al., 2002). In mammals, AIF, a large flavin-adenine dinucleotide binding oxidoreductase protein, was

shown to be released upon death induction, in a caspase independent but Bcl-2 dependent manner, upon which it promotes cell death through DNA degradation (Susin et al., 1999; Joza et al., 2001). However, it remains unclear whether the release of AIF is truly caspase independent since it has been reported that Z-VAD-FMK, a caspase inhibitor, could inhibit the release of AIF, but not of Cytochrome *c*, from mitochondria following intrinsic death stimuli (Arnoult et al., 2003). Similar to *cps-6*, in *wah*-1 RNAi embryos cell death progression was delayed and partially blocked (Wang et al., 2002). This phenotype could be easily explained by compromised mitochondrial respiration, presuming *wah-1* has an effect outside of cell death, and not through a specific cell death defect. Furthermore, the evidence suggesting both the release after death induction and the mitochondrial localization of *wah-1* should not have been concluded since the microscopy was not correctly performed. Further investigations are required to clearly demonstrate a role if any for either *cps-6* or *wah*-1 in *C. elegans* cell death. As of yet there is very little evidence that apoptogenic factors are released from the IMS of mitochondria, however, this little evidence does support the hope that conservation from worm to man is greater than previously suspected.

1.4 Execution phase of programmed cell death in *C. elegans*. Is the picture complete?

As mentioned above (Fig.1a), three *C. elegans* genes, *egl-1*, *ced-4*, *ced-3*, are required for all somatic programmed cell death to occur. In cells destined to live, CED-9 is required to protect against cell death by inhibiting CED-4 through sequestration to the mitochondria.

Thereby, preventing CED-4's release into the cytosol and thus inhibiting its pro-apoptotic function of stimulating CED-3 activity. Is this simple genetic model complete? As mentioned in the above sections, mitochondria could be more important in cell death and require a more specific investigation (see section 1.3). Furthermore, reasons to suggest that pieces of the puzzle are still missing come from other lines of investigation.

1.4.1 Overexpressed *egl-1* kills cells

Besides epistasis experiments, transcriptional overexpression has also been used to delineate the genetic pathway seen in Fig. 1. Of interest, overexpressing EGL-1, CED-4 and CED-3 causes killing of cells that normally live. If the pathway holds, EGL-1 and CED-4 induced killing is completely blocked in *ced-3* mutants. This is not the case, albeit inefficiently, increased death occurs in these transgenic embryos (Shaham and Horvitz, 1996a; Abraham and Shaham, 2004). This raises the following question: Can overexpressed EGL-1 and CED-4 trigger programmed cell death independently of CED-3? Of interest, it has been reported, that a small but detectable number of cells randomly die in animals that completely lack *ced-3* activity, indicating that there might exist a *ced-3*-independent or even caspase-independent death program during *C. elegans* development (P. Reddien, F. Xie and H.R. Horvitz, unpublished results). Could this be an uncharacterized programmed cell death pathway in *C. elegans* required in the elimination of compromised, virus-infected, or cancerous cells?

It has been proposed that alternative cell death pathways could be mediated by the existence of three other caspase-like genes in the worm genome, *csp-1*, *-2* and *-3*, which

encode six different proteins (Shaham, 1998; Abraham and Shaham, 2004). Caspases are not necessarily executioners of cell death. They have been shown to have roles outside of programmed cell death, such as in *D. melanogaster* sperm formation (Arama et al., 2003). It is likely that *csp-1*, *-2* and *-3* could have role outside cell death since it was reported that silencing of *csp-1*, *-2* and *-3* does not block cell death (Abraham and Shaham, 2004). This is not to mean that maybe novel, uncharacterized cell death events are not mediated through these putative caspases.

Furthermore, the killing initiated by EGL-1 has been suggested to be far more effective than that induced by CED-4 (Ravi Jagasia, unpublished observation). This suggests that EGL-1, the initiator of the signal transduction cascade, could be recruiting and/or subsequently activating other components to amplify the core cell death pathway. What are these components? It could be through a mitochondrial pathway. This hypothesis was an important starting point for the experiments presented in this work.

1.4.2 *ced-9*, dual roles

Genetic data suggest that, in addition to its death-preventing role, CED-9 can also have killer promoting function (Hengartner and Horvitz, 1994b). It was reported that loss-of-function mutations in *ced-9* lead to enhanced rather than diminished cell survival in mutants slightly reduced in *ced-3* and *ced-4* function (Hengartner and Horvitz, 1994b; Shaham and Horvitz, 1996b). This has precedent in the mammalian world; *ced-9* homologues *bcl-x* and *bcl-2* each encode pro- and anti-apoptotic forms (Boise et al., 1993; Cheng et al., 1997). However, unlike in mammals, there is no evidence that *ced-9*

transcript is alternatively spliced or that the CED-9 protein is proteolytically processed, as is the case for *bcl-x* and *bcl-2*, respectively. It appears that CED-9 pro-apoptotic function only becomes unveiled during the process of cell death. What could this killing function be? Could it have again something to do with the mitochondria? As postulated above, it could confer a Bax like activity, possibly through formation of a channel in the mitochondrial outer membrane, since the two proteins are structurally similar. However, this may not have anything to do with CED-9 directly. An alternative reason stems from the fact that CED-9 recruits CED-4 to the mitochondria. In a *ced-9(lf)* background, CED-4 is mislocalized to the perinuclear membranes (Chen et al., 2000); this could render it not as effective at killing, since it could possibly acquire an additional partner or processing step while sequestered to the cytoplasmic leaflet of the outer membrane of the mitochondria. Further evidence is needed to suggest a direct role of CED-9 in killing. In light of these observations the model is certainly incomplete. Whatever the killing function of CED-9 is, be it direct or indirect, it is likely occurring after the initiation of death, during the process of dying at the mitochondria.

1.4.3 *icd-1*

Another observation suggesting that this story is incomplete comes from an RNAi screen looking for regulators of early embryogenesis. A gene was identified, named inhibitor of cell death-1 (*icd-1*), which, as its name implies, inhibits cell death. Silencing of ICD-1, through RNAi, led to excessive death in embryos (Bloss et al., 2003) which was suppressed in *ced-4* but not *ced-3* mutant animals. This led to the tempting possibility that a new pathway that is independent of the caspase CED-3 was uncovered through this

RNAi screen. On first glance, this seems counterintuitive since, except for the leader cell of the male gonad; all somatic cell death events require active CED-3. If silencing of ICD-1, leads to ectopic CED-4-dependant cell death it would suggest that a pathway within all cells of the embryo exist that is independent of the functional caspase which has not previously been functionally characterized. Or maybe what was being observed was not at all programmed cell death. In fact this was determined during the course of the present study (Jagasia et al., 2005). ICD-1 encodes for the beta-subunit of the human nascent polypeptide-associated complex (betaNAC) and its *E. coli* homologue, trigger factor. The NAC is the first cytosolic protein to contact nascent polypeptide chains emerging from the ribosome (Lauring et al., 1995; Reimann et al., 1999; Beatrix et al., 2000), and it is important in correct protein folding and transport of the newly synthesized polypeptides. It is likely that the effects seen after *icd-1*(RNAi) silencing are non-specific due to a complete misfolding and mistargeting of thousands of newly translated proteins.

1.4.4 The current model is incomplete

To conclude, the study of *C. elegans* programmed cell death is not saturated, many of the players involved are unknown and burning questions remain to be addressed. As outlined in sections 1.3 and 1.4, many unexplained observations point to the mitochondria having a greater importance in *C. elegans* programmed cell death. It will require novel approaches, such as in *vitro* reconstitution and *in vivo* imaging, to directly answer some of the questions, which classical genetics have failed to elucidate. A big hole is present as

to the role of mitochondria in *C. elegans* programmed cell death. This project attempts to shed light on some of these gaps.

1.5 Mitochondrial morphological dynamics

In text books mitochondria are generally depicted as static round or oval shaped organelles. With the emergence of new fluorescent vital dyes (see Molecular Probes: The Handbook. Chapter 12) and mitochondrially targeted fluorescent proteins (De Giorgi et al., 1999; Westermann and Neupert, 2000), it has gradually become clear that the morphology of mitochondria is highly variable, dynamic and complex. In the past few years and during the course of this work, significant advances were made in the study of the mechanisms that regulate mitochondrial dynamics (Rube and van der Bliek, 2004). These advances were stimulated because mitochondria are universally important for cells, involved indirectly in all cellular processes and have directly been linked to apoptosis (Section 1.3) , ageing (Dillin et al., 2002; Trifunovic et al., 2004), calcium regulation (Pinton et al., 1998), cell development (Hales and Fuller, 1997; Labrousse et al., 1999) and plasticity (Li et al., 2004). The term mitochondrial dynamics refers to the mechanisms that regulate both mitochondrial morphology and cellular distribution.

From initial morphological studies in *C. elegans*, in yeast, and in mammalian cells it became clear that mitochondrial morphology is not random, but highly coordinated and determined by cell type most likely through the cell's mitochondrial requirements (Damsky, 1976; Labrousse et al., 1999; Collins et al., 2002). For instance, in *C. elegans*

muscle cells individual mitochondria are arranged in tracks that are parallel to the actin/myosin filaments (Labrousse et al., 1999), in germ line cells a single mitochondrion is arranged around the nucleus (Labrousse et al., 1999), in neurons mitochondria are arranged both around the soma, while being dispersed in axons and again concentrated in dendrites (Ravi Jagasia, unpublished observation), and in embryonic cells mitochondria appear as tubular network like structures (Jagasia et al., 2005). The specific morphology of mitochondria in a particular cell, whether a network or disconnected, will have specific consequences for the cell as well as the organelle. For example, Ca^{2+} waves through mitochondria, which enable fast intra-organelle communication, are depressed in fragmented mitochondria (Szabadkai et al., 2004). Furthermore, slight fluctuations in mitochondrial membrane potential will not be transmitted in fragmented mitochondria due to loss of electrical coupling. So why would mitochondria be fragmented in a cell or exist as discrete entities? The likely reason is that discrete subpopulations of mitochondria are required to serve discrete functions exemplifying the multifunctionality and plasticity of the organelle.

In yeast, mitochondria divide and subsequently fuse again in the order of minutes (Nunnari et al., 1997; Jakobs et al., 2003a). Mitochondrial morphology is dictated by the balance of these two opposing processes, fusion and fission. When fusion predominates mitochondria appear as interconnected or networked. Conversely, when the balance is tilted to fission, mitochondria display a more fragmented phenotype. These two events are coordinated by complex proteinaceous machines. Several components have been identified by recent genetic screens in *S. cerevisiae* (Dimmer et al., 2002), unraveling the

important players in these two processes. However, although a great deal of components of the core fusion and fission machineries have been identified and a fuzzy picture of how the processes proceeds mechanistically is emerging very little is known about what regulates this machinery and to what extent signal transduction is involved. As well, considering the fast and constant kinetics of fusion and fission events, as of yet no real function has been elucidated for the dynamics of mitochondrial morphology.

Because of early morphological descriptions of apoptosis, it was believed that mitochondria remain unchanged during cell death (Kerr et al., 1972). This view has slowly changed with the observation, exciting to mitochondrial morphologist and forming the basis of this work in *C. elegans*, that mitochondrial morphology appears to change during apoptosis and that these changes might be required for apoptosis to proceed (Frank et al., 2001; Breckenridge et al., 2003). Mitochondrial fragmentation was originally observed in mammalian cells grown in culture upon exposure to a variety of death inducing stimuli, such as tyrosine kinase inhibitors (Mancini et al., 1997), etoposide, a DNA topoisomerase II inhibitor (Dinsdale et al., 1999), cycloheximide (Dinsdale et al., 1999), NGF deprivation (Martinou et al., 1999), Bax transfection (Desagher and Martinou, 2000), and staurosporine (Frank et al., 2001). As well, in various hyper-apoptotic neurodegenerative diseases mitochondria appear round, higher in number and ultracondensed (Trimmer et al., 2000; Menzies et al., 2002; Ferreirinha et al., 2004; Trimmer et al., 2004). Could this transition to a more fragmented mitochondrial state be required for cell death? Because of this tempting idea this study investigated

what happens to the morphology of mitochondria during physiological cell death in *C. elegans*.

1.6 Mitochondrial fission

Mitochondrial fission has been shown to be required in dividing cells to ensure inheritance of mitochondria by daughter cells and in post mitotic neurons to mobilize mitochondria at synapses for the achievement of plasticity (Li et al., 2004; Schuman and Chan, 2004). Outer mitochondrial membrane fission has recently been linked to control by signal transduction (Alto et al., 2002; Pozniakovsky et al., 2005), Ca^{2+} (Breckenridge et al., 2003), sumoylation (Harder et al., 2004), and cAMP (Alto et al., 2002).

In yeast, where the function of mitochondrial fission has been best studied (Shaw and Nunnari, 2002), mitochondrial outer membrane fission appears to be regulated by three core components, the cytosolic proteins Dnm1p and Mdv1p, and Fis1p which spans the outer mitochondrial membrane. Dnm1p is a guanosine triphosphatase (GTPase) related to dynamin, which has a similar GTPase domain, dynamin-2 domain and GED domain (Bossy-Wetzel et al., 2003). The mechanism, by which Dnm1p mediates fission, is still a matter of debate. Dynamin protein most likely contributes a physical force to the breaking of the outer membrane through rounds of GTP hydrolysis and/or recruits proteins into the complex, which then acts to severe the outer membrane (Fig.2a and b). The *MDV1* gene encodes a soluble 80 kDa protein with an N-terminal extension, a central coiled-coil domain and seven C-terminal WD repeats. Mdv1p has been shown to

regulate but not recruit Dnm1p into specific clusters and to somehow stimulate Dnm1p mediated fission (Tieu and Nunnari, 2000) (Fig. 2a and b). The 17 kDa, Fis1p protein spans the outer membrane, with its N-terminus in the cytoplasm and its C-terminus protruding into the inter membrane space. Fis1p recruits initially Dnm1p to mitochondrial membranes, regulates its assembly probably via direct interaction with Mdv1p, and then stabilizes this complex on the cytoplasmic face of the mitochondria (Tieu et al., 2002). As highlighted in Figure 2, it is completely unknown how the event is initiated (red) from the cytoplasm or to what extent outer and inner membranes coordinate through cross talk (aqua marine).

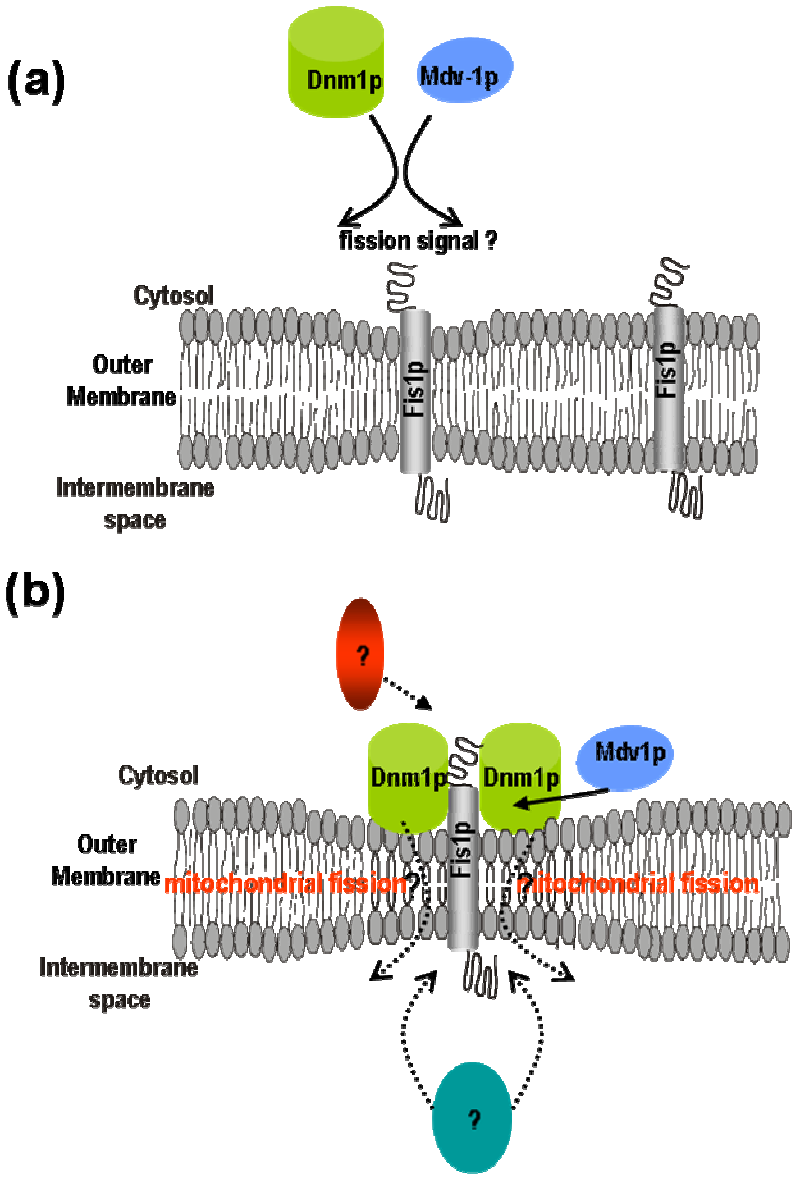

Figure 2. Overview of mitochondrial fission in yeast, what is known and unknown.

(a) Dnm1p and Mdv1p reside in the cytoplasm but, upon activation by unknown signals (red question mark), they both translocate and bind to the mitochondrial outer membrane via Fis1p (b) At the site of complex assembly, Dnm1p, like dynamin, could potentially assemble into an oligomeric complex forcing the severing of the membrane and/or involved in the recruitment of yet unknown factors to the complex, depicted in red. Whether cytoplasmic, inter membrane space or inner membrane proteins regulate outer membrane fission is unknown (displayed as a red question mark and aqua marine question marks, respectively). Abbreviations: DNM-1, dynamin protein 1; MDV-1, mitochondrial division 1, FIS1, fission 1 (Shaw and Nunnari, 2002; Bossy-Wetzel et al., 2003).

Dnm1p homologues identified and determined to control mitochondria fission are mammalian Drp1 (Smirnova et al., 1998), and *C. elegans* DRP-1 (Labrousse et al., 1999). Homologues of Fis1p have been identified in humans, hFis1 (James et al., 2003) , and based on sequence identity, in *C. elegans* (Two putative *C. elegans* candidates were identified in the course of the present work and named, *fis-1* and *fis-2.).* So far no Mdv1p homologue has been found in higher eukaryotes; however it could be possible that another gene, endophilin B1 in humans and *erp-1* in *C. elegans,* may have replaced its function. Like Mdv1p in yeast, endophilin B1 in mammalian cells, is required for mitochondrial fission and acts downstream of Drp-1 (Karbowski et al., 2004). *erp-1* may have a role in *C. elegans* mitochondrial fission (Dr. van der Bliek, personal communication).

A basic pathway for mitochondrial fission is known, however, many questions require further attention. To address this will require most certainly the development of *in vitro* assays to reconstitute and dissect the individual steps in the fission pathway. A burning issue, as of yet unresolved, is whether mitochondrial outer and inner membrane division is coordinated. In yeast it was thought to be coupled (Bleazard et al., 1999; Tieu and Nunnari, 2000). However in *C. elegans*, interfering with *drp-1* function blocked outer mitochondrial membrane fission while division of inner membrane remained constant (Labrousse et al., 1999). It is conceivable that blocking outer membrane fission could impede inner membrane fusion, thus the matrix would appear normal since the balance had returned. Recently, inner and outer membrane fission was genetically dissected in

yeast. Mdm33p was found to be required for mitochondrial inner membrane fission and was placed upstream of Dnm1p, suggesting that mitochondrial inner membrane division precedes mitochondrial outer membrane division (Messerschmitt et al., 2003). These two studies suggest that the processes of fission are not tightly coupled and that the inner membrane can divide independently. These results are hard to reconcile, since recent work has demonstrated that many cytosolic factors such as Ca^{2+} (Breckenridge et al., 2003), protein kinase A (Alto et al., 2002) and SUMOylation (Harder et al., 2004) regulate fission through the cytosolic dynamin, most likely by controlling its translocation and stimulation at the cytoplasmic leaflet of the outer mitochondria membrane. What sense would mitochondria have for the inner membrane to divide independently?

These studies on the mitochondrial fission machinery have relied mostly on genetic and biochemical data and owing to the dynamic nature of mitochondria, important aspects probably have been overlooked. Detailed studies analyzing mitochondrial dynamics resolved in time and space strongly suggest that outer and inner membranes fission appear to be tightly coupled and likely occur concomitantly (Jakobs et al., 2003b; Jakobs et al., 2003a). It appears that an early event during mitochondria fission process is the constriction of the matrix (Legesse-Miller et al., 2003). Using markers for both outer-mitochondrial membrane and matrix, discontinuity or constriction of the matrix was never seen without a discontinuity of the outer membrane (Jakobs et al., 2003a). Furthermore, it appears that both Dnm1p and Mdv1p are partially required for the constriction of the matrix and the proceeding fission event (Astrid Schauß and Stefan

Jakobs, Personal communication). In light of these results, it appears that both outer and inner membrane fission are very tightly coupled and that these events likely do not occur independently.

1.7 Mitochondrial fusion

Mitochondrial membrane fusion is complex; it is the coordinated process of fusing four membranes. Fusion of mitochondria serves to merge discrete mitochondria in order to mix and thus complement their individual components. In yeast and human cells, it has been demonstrated that this is important for the transmission of mitochondrial genomes from the mother to the dividing cell (Westermann, 2002, 2003). Since, through mitochondrial membrane fusion, cells lacking mitochondria DNA (mtDNA) can be rescued it has been thought that the intermixing of mtDNA is important consequence of the process of fusion. It has been proposed that the fusion of mitochondria is required for organisms over the lifespan to rescue through trans-complementation the accumulation of somatic mutations in the mitochondrial genome. This is unlikely to be the only reason for mitochondrial fusion. When one considers the kinetics, fusion and fission is occurring rapidly and frequently, in yeast one event per two minutes (Nunnari et al., 1997), However, mutations occur slowly, so why would the mitochondria need to be so dynamic? As well, trans-complementation would not be required if mitochondria maintained an intracellular mitochondrial continuum, which is seen in many mammalian cells. Is then the sole function of fusion to counterbalance the opposing process, fission? Probably not. As with fission, many burning issues remain unanswered.

Until recently, we could only speculate about the molecular mechanisms underlying mitochondrial membrane fusion, since a system reconstituting fusion *in vitro* did not exist. Recently it was established that outer and inner membrane fusion can be separated, suggesting a two step process, since intermediary complexes exist, in which outer but not inner membrane appeared fused (Meeusen et al., 2004). Furthermore, the fusion of both inner and outer membrane required GTP hydrolysis (Meeusen et al., 2004) which is not surprising, since two GTPases were previously found to regulate fusion, Fzo1p and Mgm1p (Rapaport et al., 1998; Wong et al., 2003).

Fzo1p and Mgm1p are highly conserved large GTPases. They are components of the mitochondrial outer and inner membranes, respectively (Rapaport et al., 1998; Wong et al., 2000). The Fzo1 protein has been functionally characterized in *S. cerevisiae,* in *D. melangaster*, the organism of discovery (Hales and Fuller, 1997), and in human cells, where two homologues exist, MFN 1 and 2 (Chen et al., 2003) In addition, a previously uncharacterized homologue has been found in the *C. elegans* genome, *fzo-1*(Westermann, 2003). Yeast lacking Fzo1p have highly fragmented mitochondria and lose their mitochondrial DNA (Hermann et al., 1998; Rapaport et al., 1998). In mice, *mfn1* or *mfn2* knockouts are embryonic lethal and primary cells contain highly fragmented mitochondria that are unable to fuse. In yeast, Mgm1p is also required for mitochondrial fusion (Wong et al., 2000; Wong et al., 2003). It is localized in the inter membrane space. Mgm1p has a homologue in humans, *OPA1,* and in *C. elegans,* EAT-3/MGM-1. The precise role of Mgm1p in mitochondrial fusion is still unknown.

However, since it is localized to both the inner membrane and the inter membrane space, it is likely involved in inner membrane fusion. In yeast, Mgm1p exists in two forms of different lengths, short and long, found in the inter membrane space and the inner membrane respectively. Both forms are required for maintaining mitochondrial morphology (Herlan et al., 2003).

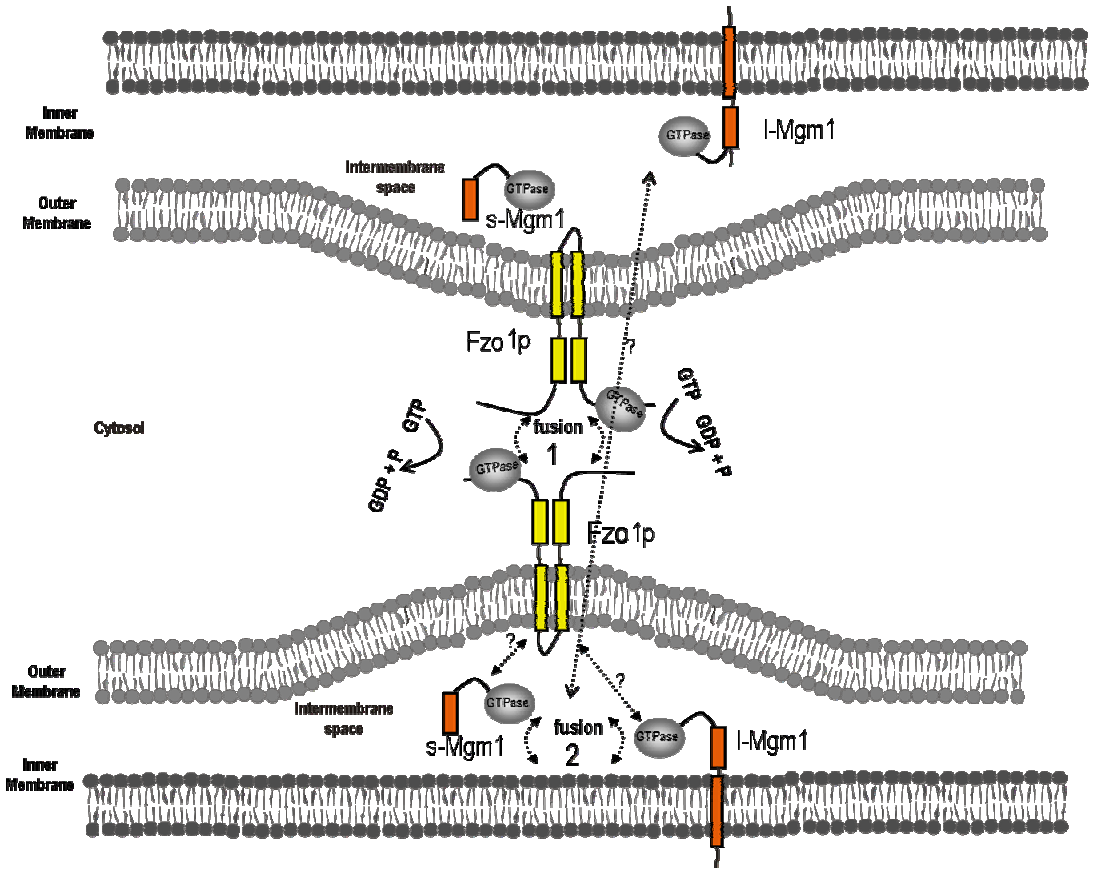

Figure 3. Overview of a simplified, two step, mitochondrial fusion in yeast, what is known and unknown.
Mitochondrial fusion of outer membrane precedes fusion of inner membrane. Fzo1p regulates fusion of the outer membrane. Mgm-1p exists in two forms, s-Mgm-1 and l-Mgm-1, and likely coordinates inner membrane fusion. Step 1 of mitochondrial fusion involves docking of two mitochondria, through their respective outer membranes, mediated through Fzo1p, through a homotypic trans interactions. Step 2 of mitochondrial fusion proceeds after the completion of Step 1, involves fusion of the two mitochondrias' inner membranes. This process likely requires both forms of Mgm-1p, to what extent is still unclear, and whether as well through homotypic trans interactions is unknown. How and to what extent the two membranes communicate, is completely unknown. Abbreviations: Fzo1p, fuzzy onions; s-Mgm-1, short mitochondrial genome maintenance form; l-Mgm-1, long mitochondrial genome maintenance form; GTP, guanosine triphosphate; GDP, guanosine diphosphate; P, phosphate; GTPase, guanosine triphosphate hydrolase. (Bossy-Wetzel et al., 2003; Westermann, 2003; Meeusen et al., 2004)

As is the case for fission, very little is known how and why mitochondrial organelles fuse. Some recent yeast work worth mentioning has suggested that the process may be regulated by the steady state levels of Fzo-1p controlled through the 26S proteasome (Fritz et al., 2003; Neutzner and Youle, 2005). Fzo-1p levels drop during pheromone-induced mating response mediated through signal transduction of the MAP kinase pathway (Neutzner and Youle, 2005). Of interest, high levels of this pheromone induce yeast death (Severin and Hyman, 2002) and this has recently been shown to be mediated by mitochondrial fragmentation (Pozniakovsky et al., 2005). Could this be the evolutionary roots of cell cycle control and apoptosis, through mitochondrial morphology?

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1.8 Mitochondria fission, fusion and apoptosis

To reiterate, mitochondria have been shown to fragment upon exposure to death-inducing stimuli. This has been shown for numerous organisms, including mammals (Esseiva et al., 2004; Hardwick and Cheng, 2004)) and yeast *S. cerevisiae* (Hardwick and Cheng, 2004). Mitochondrial morphology is a balance between fusion and fission. The fragmented phenotype after exposures to death stimuli could arise from either stimulation in fission or inhibition of fusion. Since, an early event in some cell death events is a loss of mitochondrial membrane potential (Zamzami et al., 1995b; Zamzami et al., 1995a), and a loss of potential across the mitochondria impedes mitochondrial fusion (Legros et al., 2002) it could be possible that this fragmentation phenotype was simply an indirect

consequence of a steep drop in potential. This would suggest that fission is not required for cell death. However this is not the case.

A link between mitochondrial fission and apoptosis was found. After induction of cell death, it was observed that human dynamin-related protein, Drp1, translocated from the cytosol to mitochondria (Frank et al., 2001). Furthermore, overexpressing Drp1 (dominate negative(dn)) caused a block of mitochondrial fission, Cytochrome *c* release, and indeed a delay in cell death itself (Frank et al., 2001). This provides a direct link between the fission phenotype and cell death. However, it was not shown whether mitochondrial fission alone could induce cell death, and thus, if the fragmentation is caused by stimulation of fission or inhibition of fusion. Emerging reports using mammalian cell culture systems suggest that both can trigger apoptosis.

Supporting fission inducing apoptosis, it was shown that overexpression of hFis1, the human orthologue of yeast FIS1, induced mitochondrial fragmentation and Cytochrome *c* release (James et al., 2003) and this event could be blocked by introducing Drp1(dn). However, very little can be concluded from this work, the effects were not observed in physiological time periods. Specifically, Cytochrome *c* release and cell death were only seen 36 hours after the introduction of hFis1 whereas fragmentation occurred after 16 hours. Apoptosis is rapid, occurring within minutes after introduction by cell-death stimuli, such as Fas (Kawahara et al., 1998b; Kawahara et al., 1998a), TNF (Robaye et al., 1991) or Bax dimerization (Gross et al., 1998). It is conceivable that the effects reported for hFis1 are only secondary to a general toxicity due to mitochondrial

impairment. The logical experiment would be to demonstrate a role for hFis1 in apoptosis. For example determine whether depleting hFis1 function could have an effect on blocking apoptosis after physiological induction to die. This analysis was recently performed in mammals and yeast (Fannjiang et al., 2004; Lee et al., 2004) and its role in cell death is presently unclear.

Evidence supporting a block in fusion causing the induction of apoptosis comes from two converging approaches, human mitochondrial genetics and mammalian cell culture. Human geneticists have linked the two known mitochondrial GTPases involved in fusion to degenerative diseases, caused possibly by excessive apoptosis. 1) Mutations in *OPA1*, the Mgm-1p homologue, lead to dominant optic atrophy, a human disease characterized by loss of retinal ganglion cells (Alexander et al., 2000; Delettre et al., 2002). 2) The human Fzo-1p homologue, MFN-2 is mutated in patients with Charcot-Marie-Tooth disease type 2a (Zuchner et al., 2004; Kijima et al., 2005). From experiments with cell culture systems, roles for the two GTPase in apoptosis were recently demonstrated. Knocking down *OPA1* function using RNA interference induces Cytochrome *c* release and subsequently cell death (Olichon et al., 2003). Overexpression of active Fzo1 inhibited both Cytochrome *c* release and activation of Bax/Bak (Neuspiel et al., 2005). Furthermore, silencing the Fzo1 gene sensitized cells to apoptosis stimuli (Sugioka et al., 2004).

As of yet, mammalian studies have failed to distinguish whether fission or fusion are regulated in cell death, most likely because both are involved. Evidence is emerging that

multiple components of both processes are involved in apoptosis and can positively and negatively regulate it (Lee et al., 2004). This model is further supported by the observation that Bax can colocalize with both Drp1 (fission component) and Mfn2 (fusion component) at discrete patches on the mitochondrial outer membrane (Karbowski et al., 2002).

1.9 Aim of this thesis

Since mitochondrial fragmentation occurs and is required for cell death in both mammals and unicellular eukaryote, it might also be involved in *C. elegans* physiological cell death. As mentioned above, until now the evidence supporting a role for mitochondria in *C. elegans* apoptosis is meager at best. Thus the initial aim of this work was to address the importance of mitochondria in *C. elegans* PCD. It was hypothesized that by observing mitochondria *in vivo* during cell death, the role, if any, of mitochondria in cells destined to die in *C . elegans* could be better understand.

1.9.1 Observation of mitochondria *in vivo* during PCD

In order to study mitochondria during *C. elegans* cell death, two ways were established to observe mitochondria prior to or at the time of the initiation of physiological cell death in *C. elegans*. This allowed a qualitative measure of three distinct properties of mitochondria during cell death: 1) mitochondrial morphology, 2) mitochondrial membrane potential, 3) mitochondrial protein import. All three of these mitochondrial

properties have been shown to be affected during cell death in mammalian cells (Zamzami et al., 1995b; von Ahsen et al., 2000; Frank et al., 2001).

1.9.2 Determine if/what components of the cell death machinery are involved in mitochondrial fragmentation

It was then asked whether mitochondrial fragmentation occurs in *C. elegans* mutants defective in the components of the core cell death machinery. This was done to address whether mitochondrial fragmentation is a consequence of the effector function of the active caspase, CED-3, or plays a role prior to caspase activation.

1.9.3 Determine whether mitochondrial fragmentation is required and sufficient for PCD

There could be numerous etiologies of the observed mitochondrial fragmentation such as a) the stimulation of the fission apparatus, b) the inhibition of the fusion apparatus, c) or maybe a general breakdown of respiring mitochondria causing a loss mitochondrial integrity. To address this, three general approaches were taken: RNAi knockdown of genes involved in fusion and fission, construction of deletion strains of genes involved in fusion and fission, and temporal expressing both wild-type and mutant forms of proteins involved in fusion and fission.

2 Results and discussion

2.1 Morphology of mitochondria in cells undergoing programmed cell death

When mitochondria are visualized in living embryos, the organelles are distributed evenly throughout the embryo (Fig. 9). Mitochondria within a cell appear as a tubular network (Fig. 9). To monitor the morphology of mitochondria during physiological cell death *in vivo,* transgenic worms were generated expressing GFP targeted to the mitochondrial matrix (*mitogfp*, the mitochondrial import signal of chicken aspartate aminotransferase fused to GFP). This fusion protein was previously shown to successfully label mitochondria in body wall muscles when expressed under the control of the muscle-specific *myo-3* promoter (Labrousse et al., 1999). To induce the expression of *mitogfp* during embryonic development, two different *C. elegans* promoters were used, a heat-inducible promoter or the *egl-1* promoter. The expression of the transgenes was combined with labeling of the mitochondria with rhodamine, a voltage sensitive dye, previously used to study the distribution and morphology of mitochondria in the *C. elegans* embryo (Badrinath and White, 2003) and in adult *C. elegans* gonads (Labrousse et al., 1999).

Using the two different promoters, two approaches were devised to monitor mitochondria during cell death: 1) *egl-1*, which is known to cause ectopic programmed cell death (Conradt and Horvitz, 1998), was ectopically expressed simultaneously with *mitogfp* during embryogenesis. In order to achieve this, *egl-1* and *mitogfp* were placed under the

control of the heat shock promoter (referred to as P_{hs}*egl-1* and P_{hs} *mitogfp,* respectively), which is known to have robust expression during embryogenesis, the developmental time period, during which most cell death events occur (Stringham et al., 1992). 2) Physiological cell death events were observed by expressing *mitogfp* under the control of the *egl-1* promoter (referred to as P_{egl-1}*mitogfp)*. *egl-1* appears to be specifically expressed in cells destined to die (Conradt and Horvitz, 1999). Therefore, *mitogfp* should only be expressed in cells that normally die. Both approaches have advantages and limitations. Approach 1 is an effective and fast way to induce cell death; however, it is caused by ectopic expression of *egl-1*. Thus cells that normally survive are committed to die, and the effects observed may not necessarily be physiological. In approach 2, physiological cell death events are observed; however, at any time during embryogenesis only a few cell deaths occur. In addition, using either one of the approaches, it is unclear how far along the cell death process has proceeded by the time the GFP signal appears since *egl-1* is concomitantly expressed.

2.1.1 Approach 1: P_{hs}*mitogfp* and P_{hs}*egl-1* transgene

It was initially required to test whether mitoGFP would translocate and become imported into the mitochondria when expressed under the control of the heat-inducible promoters, and if so, to determine the kinetics of this process. Within 0 to 20 minutes after a 45 minute heat shock, a GFP signal was present throughout the embryo (Fig.4b, left panel).

It appeared that initially, a significant portion of the signal was cytoplasmic (Fig.4b, left panel). After 20 minutes, most of the mitoGFP had been successfully imported into the matrix, based on the fact that GFP and rhodamine staining showed identical distribution in embryos (Fig.4a, 4b middle panel). It could be concluded that upon translation of mitoGFP, prior to translocation into the mitochondrial matrix, mitoGFP had successfully folded in the cytoplasm. Approximately 2 to 3 hours after heat shock, embryos still demonstrated a strong mitochondrial signal (Fig.4b, right panel). However at this late time point, it appeared that the mitochondrial morphology was abnormal (Fig.4b, right panel). The mitochondria typically aggregated and had a circular, ring-like structure in contrast to the normal web-like structure (Fig.4a). This is somewhat reminiscent of the morphology of mitochondria in the germline (Labrousse et al., 1999). This observation was troubling since the expression of the transgene P_{hs}*mitogfp* was required to monitor changes in mitochondrial morphology after *egl-1* activation. It was important to determine whether it was the expression of the transgene P_{hs}*mitogfp* or the heat shock itself, which was causing this aberrant mitochondrial morphology. Two hours after a 45 minute heat shock, wild-type embryos not expressing the transgene contained the same aberrant ring-like mitochondrial structures based on rhodamine staining (Fig.4c, middle and right panel). It can be concluded that the heat shock alone was sufficient to cause this effect and that this effect became visible 2 hours after exposure to heat. Considering the time frame, these effects are likely mediated through proteins expressed in response to heat stress. This heat-induced effect had been previously documented in human liver mitochondria (Popinigis et al., 1973; Michea-Hamzehpour et al., 1980) and in *Neurospora crassa* (Michea-Hamzehpour et al., 1980). Therefore, I concluded that the

heat shock constructs could only be used to measure changes in mitochondrial morphology within 2 hours of the initial heat shock, since the heat-induced changes in mitochondrial morphology were not present before this time point. This limitation is not a problem since an increase in cell death can be observed within minutes after the 45 minute heat shock and subsequent expression of *egl-1*.

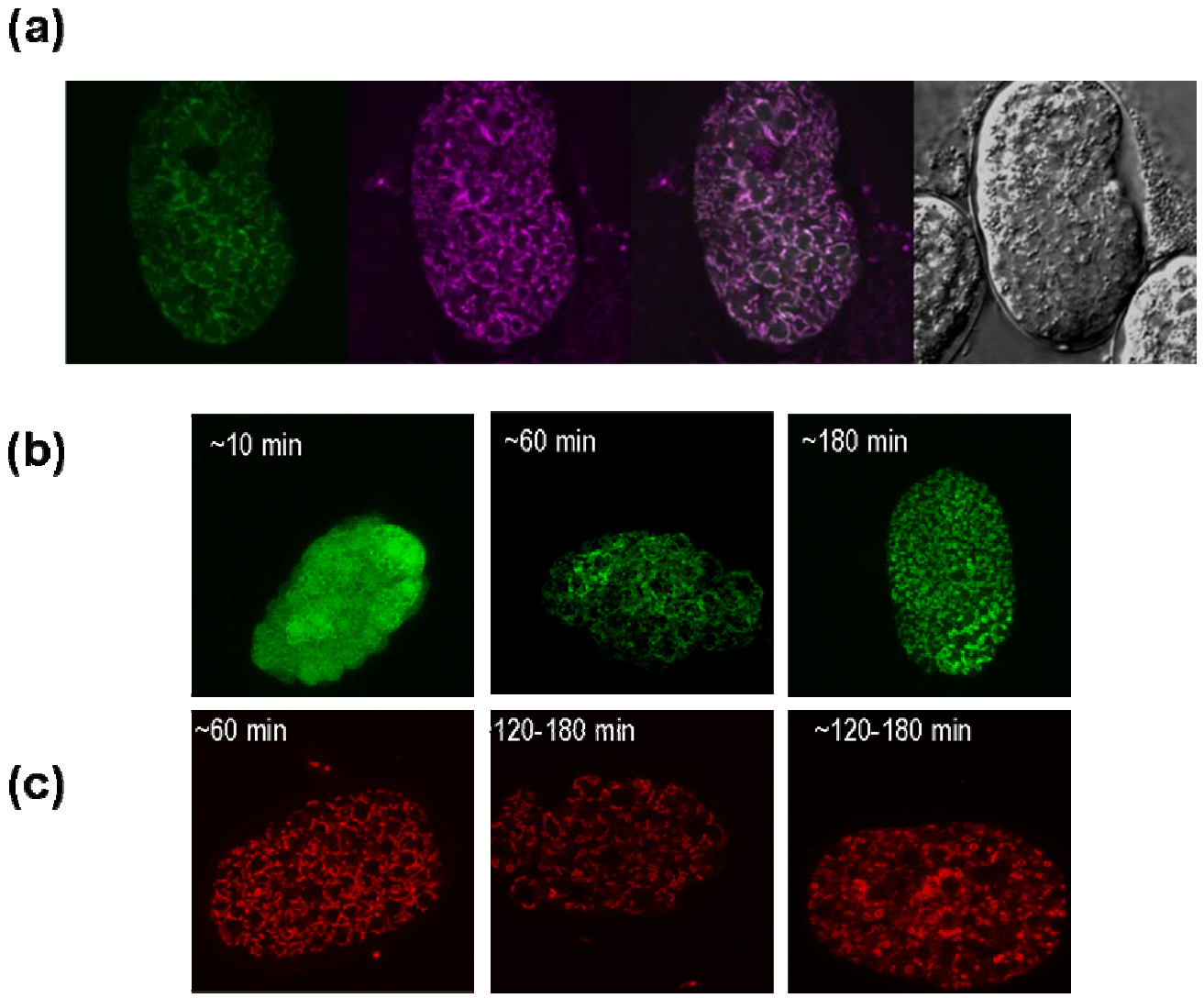

Figure 4. After heat shock mitoGFP localizes to mitochondria, but heat shock alone can induce mitochondrial morphology changes.
(a) MitoGFP localized to mitochondria 20 min after heat shock. Confocal images of GFP, rhodamine, GFP/rhodamine overlay, and DIC image (left to right) of wild-type embryos expressing P_{hs}*mitogfp* 1 hour after heat shock. (b) Kinetics of *mitoGFP* import into the mitochondria. Confocal images of GFP distribution in wild-type embryos expressing the transgene, P_{hs}*mitogfp*, around 10 min, 60 min and 180 min after heat shock (left to right). (c). Heat shock alone causes mitochondria to become round, circular and aggregated. Representative confocal pictures of rhodamine staining in different embryos about 60 min (left) and 120 min (middle and right) after heat shock.

It was then tested what the consequence is for mitochondria, if both *mitogfp* and *egl-1* are expressed under the control of the heat-inducible promoters. Because most cells expressed *mitogfp*, it was very difficult to identify mitochondria of a specific cell due to the fact that cell boundaries cannot be distinguished by DIC optics. In some cases it was possible to identify single cells for example when neighboring cells were not expressing *mitogfp* due to the mosaic nature of the embryo. This made it possible to observe the mitochondria of a particular cell concomitantly expressing *egl-1* (see Fig.5 below for a representative example). It could be observed that in embryos expressing both *mitogfp* and *egl-1*, mitochondria rapidly fragmented soon after mitoGFP labeled the mitochondria (Fig.5). This fragmentation was not seen in embryos expressing *mitogfp* alone (Fig.4a). At an early time point (0 min), mitochondria in embryos expressing the P_{hs}*mitogfp* and P_{hs}*egl-1* transgenes were indistinguishable from mitochondria in embryos expressing P_{hs}*mitogfp* alone (Fig. 4a). However, only in embryos expressing P_{hs}*mitogfp* and P_{hs}*egl-1* the mitochondria fragmented rapidly and translocated to the cortex of the cell (11 min 3 s). Fragmentation occurred prior to the appearance of a refractile "cell corpse", indicative that the cell had died. A few minutes after fragmentation had occurred, the particular cell turned into a refractile corpse (17 min 43 s) and remained a corpse for the duration of the time-lapse recording (32 min 10 s). These observations suggest that mitochondrial fragmentation is an early event during *egl-1*-induced cell death. Interestingly, based on the fact that mitochondrial labeling with rhodamine or tetramethylrhodamine ethyl ester perchlorate (TMRE) did not change over the 30 minutes of recording, it was concluded that even though mitochondria fragment, no dramatic loss of mitochondrial membrane

potential occurred. The significance of this finding will be discussed later (see section 2.1.3).

These observations indicate that global changes of mitochondrial morphology after *egl-1* induction can be effectively monitored with this approach (see section 2.2.2). However, it is hard to identify the mitochondria of single, dying cells.

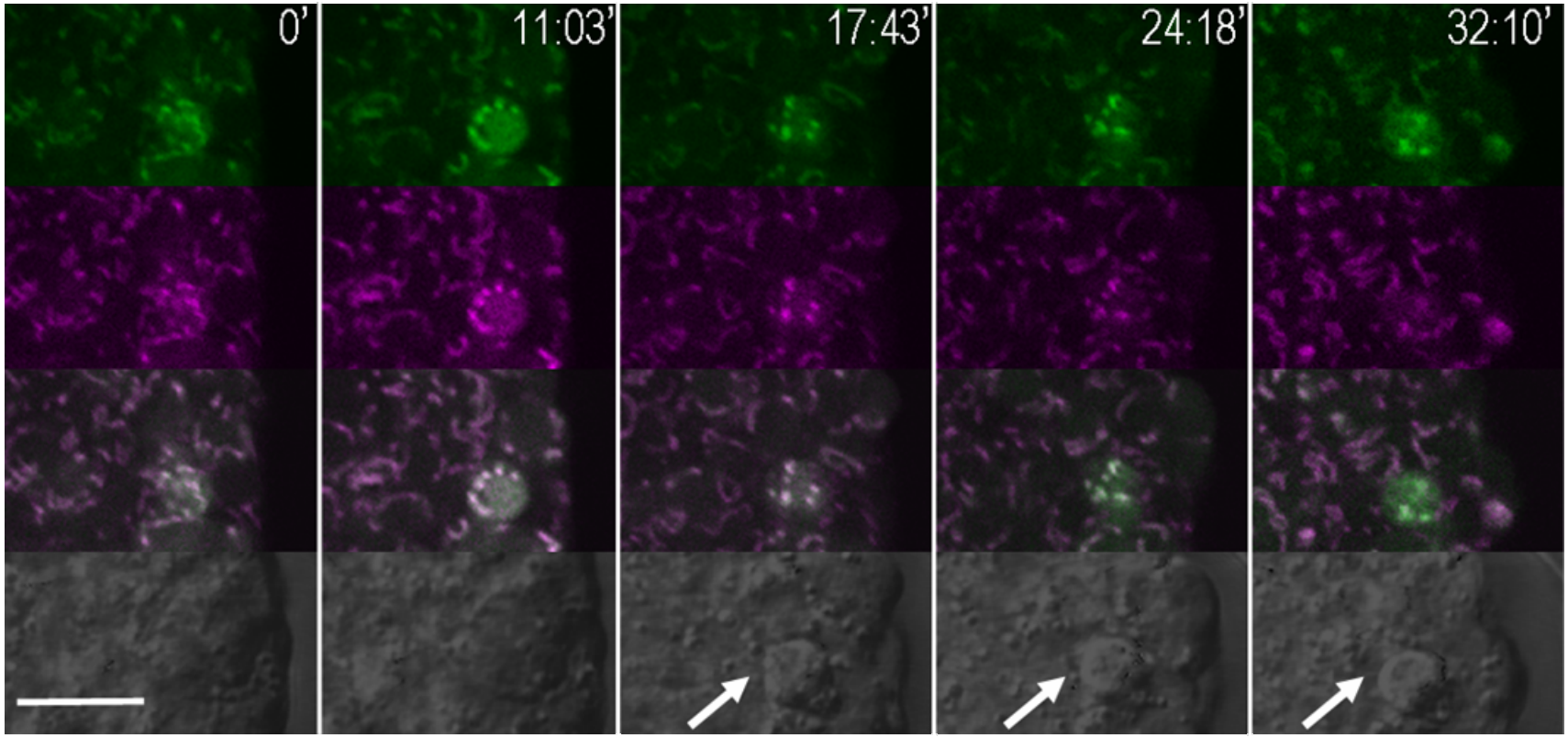

Figure 5. Mitochondrial morphology in a cell induced to undergo programmed cell death in *C. elegans* by *egl-1*.
Representative time-lapse series of confocal mitoGFP, TMRE, mitoGFP/TMRE overlay and DIC images (from top to bottom) of wild-type animals carrying a P_{hs}*mitogfp* and P_{hs}*egl-1* transgene. Images represent maximum intensity projections from four consecutive confocal image planes (0.5 µm). Scale bar, 4 µm. The arrow points to the appearance of a refractile corpse indicating that the specific cell is dead.

2.1.2 Approach 2: $P_{egl\text{-}1}$*mitogfp* transgene

The transcriptional activation of *egl-1* and the likely subsequent translocation of the EGL-1 protein to mitochondria is the earliest detectable event during programmed cell death (Conradt and Horvitz, 1998, 1999). The appearance of a GFP signal in embryos expressing the transgene $P_{egl\text{-}1}$*mitogfp* therefore marks the onset of the physiological cell death process. and this expression should be restricted only to cells destined to die (Conradt and Horvitz, 1999). Below is a representative example (n=3) of a cell in an embryo from the time *mitogfp* is initially expressed to the subsequent demise of the cell (Fig.6). After activation of the *egl-1* promoter, newly synthesized mitoGFP accumulated in the cytosol likely before being imported into the mitochondria (Fig.5). (Fig.5). This was similar to what was observed when *mitoGFP* was expressed under the control of the heat shock promoter (Fig.3b). During the initial phase of programmed cell death (i.e. shortly after the appearance of mitoGFP), the mitochondria in cells destined to die appear as tubular networks. This can be seen in the merged mitoGFP/rhodamine staining images (Fig.6, 0 min, 3rd panel from the top), in which the mitochondrial morphology in the dying cell is indistinguishable from mitochondria in healthy cells i.e. cells that do not express *mitoGFP*. The cell destined to die therefore expressing *mitoGFP* appears white in the merged mitoGFP/rhodamine panel while neighboring cells, not expressing the transgene, appear purple (Fig.6, 3rd panel from the top, 0 min). Within a short period of time, the mitochondria in the cell destined to die begin to fragment. The mitochondrial network starts to break down (8 min 52 s), eventually resulting in few clusters of mitochondrial fragments located in the periphery of the cell (16 min 8 s).

Similar to cells induced to die after *egl-1* expression (Fig.5), mitochondrial fragmentation was apparent before the apoptotic cell turned into a characteristic refractile corpse, which is indicative of a dead cell. These observations indicate that physiological cell death events can be effectively monitored with this approach

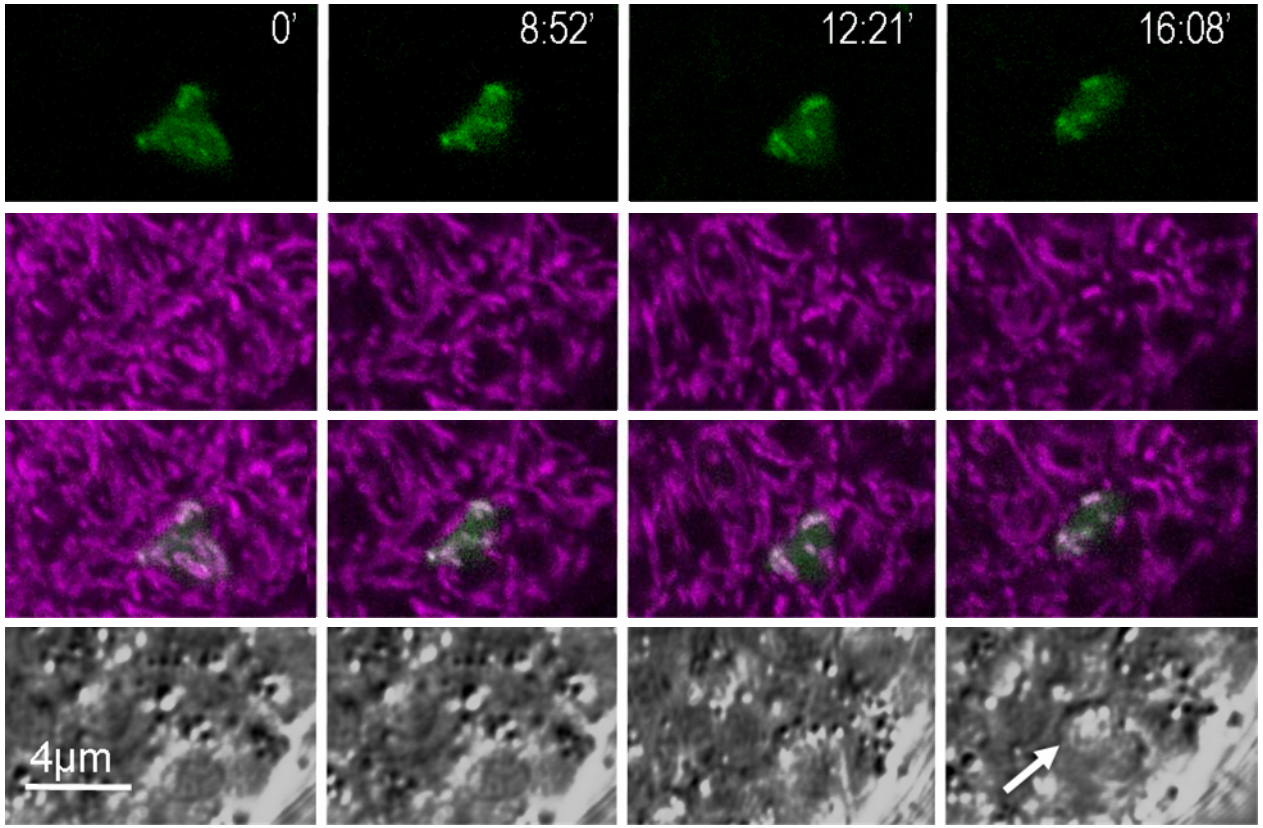

Figure 6. Mitochondrial morphology in cells undergoing physiological programmed cell death in *C. elegans*.
Representative time-lapse series of confocal mitoGFP, rhodamine, mitoGFP/rhodamine overlay and DIC images (from top to bottom) of wild-type animal carrying a stable P*egl-1mitogfp* transgene (*bcIs49*). Images represent maximum intensity projections from four consecutive confocal image planes (0.5 µm). Scale bar, 4 µm. The arrow points to the appearance of a refractile corpse indicating that the specific cell is dead.

2.1.3 Does the mitochondrial membrane potential change during apoptosis?

Since mitochondrial membrane potential has been shown to be required to maintain a mitochondrial reticulum, and because a loss of potential blocks mitochondrial fusion (Mancini et al., 1997; Mattenberger et al., 2003), it is possible that the effects observed in sections 2.1.1 and 2.1.2 are due to a loss of membrane potential. To determine whether this was the case, I examined in more detail whether there is a loss of potential during physiological or *egl-1*-induced death. In Fig. 6, it appears that there is no loss of rhodamine staining, even after the appearance of a cell corpse. However, it is possible that rhodamine is permanently sequestered through covalent modifications in mitochondria of *C. elegans* embryos. This has been documented in various mammalian cell types (Summerhayes et al., 1982; Lampidis et al., 1983). If it were true that rhodamine is binding irreversibly to the mitochondria in *C. elegans*, the vital dye could not be used to address whether there is a loss of mitochondrial membrane potential during the process of dying. Therefore, embryos were stained with the more sensitive mitochondrial membrane potential dye TMRE. This voltage-sensing dye had been previously described as one of the best mitochondrial fluorescent dyes for dynamic quantitative measurements *in vivo* because it is rapidly and reversibly taken up by live cells (Loew et al., 1993; Loew et al., 1994). Furthermore, it is far more sensitive than rhodamine at discriminating slight changes in potential (Loew et al., 1993; Fink et al., 1998).

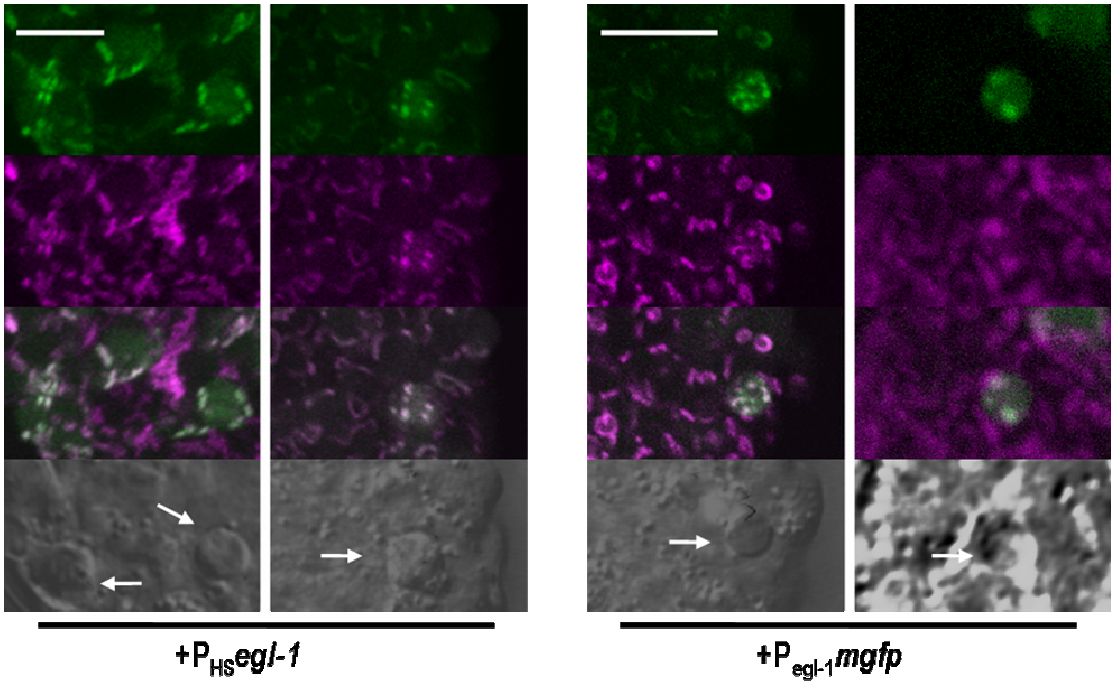

Figure 7. Mitochondrial membrane potential does not appreciably change during both physiological and *egl-1* induced cell death.
Confocal images of GFP, TMRE, GFP/TMRE overlay, and DIC images (top to bottom) of wild-type embryos expressing the transgenes P_{hs}*mitogfp* and P_{hs}*egl-1* (left) or during physiological cell death (cells expressing the transgene P_{egl-1}*mitogfp*) (right) . Arrows point to refractile corpses indicative of dead cells.

As seen in Fig. 7, based on *mitoGFP'*s expression and distribution, mitochondria are still present and fragmented in the refractile corpses. This confirms the observations that mitochondrial fragmentation occurs during cell death. In both physiological (Fig.7, right panels) and *egl-1*-induced (Fig.7, left panels) corpses, the punctate, mitoGFP-stained mitochondria co-labeled with TMRE (Fig.7). This observation suggests that the fragmented mitochondria in dead cells initially retain a membrane potential. However, in certain instances, corpses existed with punctate, mitoGFP-stained mitochondria that failed to also stain with TMRE. In order to determine at what stage the loss of TMRE staining occurred after cell death, time-lapse imaging was performed starting from the appearance of a GFP positive cell corpse (n=3). In all cases mitochondria initially appeared fragmented and retained the TMRE signal (Fig.8, 0 min). During the time of recording, the cell corpse appeared to decrease in size (Fig.8, see arrow, from 4 min 20 s to 12 min 52 s), with the concomitant loss of the TMRE signal (12 min 52 s after the appearance of a corpse). This loss in dye intensity was not due to dye bleaching since neighboring cells retained the TMRE signal.

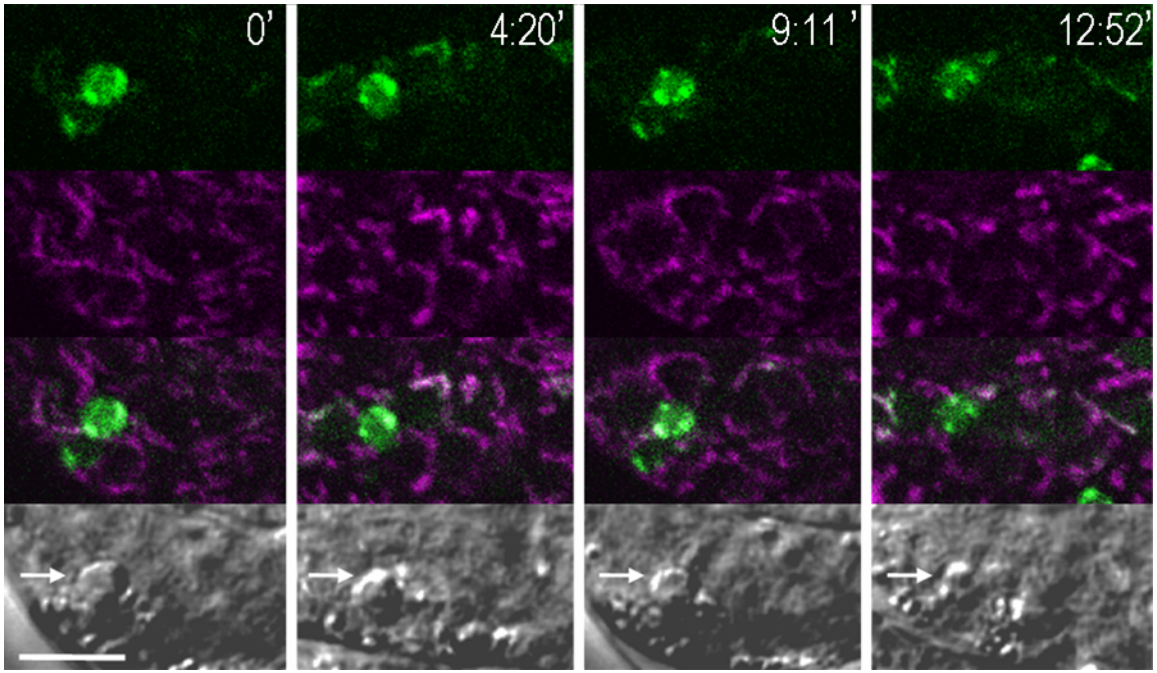

Figure. 8 Loss of mitochondrial membrane potential occurs long after the appearance of a corpse.
Representative time-lapse series of confocal mitoGFP, rhodamine, mitoGFP/rhodamine overlay and DIC images (from top to bottom) of wild-type animal carrying a stable $P_{egl\text{-}1}$*mitogfp* transgene (*bcIs49*). Images represent maximum intensity projections from four consecutive confocal image planes (0.5 μm). Scale bars, 4 μm. The arrow points to a refractile corpse.

I concluded that long after the appearance of a corpse, fragmented mitochondria within the corpse lose their membrane potential. It was not clear at what time during the cell death process this occurred. Since phagocytosis of cells undergoing apoptosis starts at an early time point, it likely occurs during the degradation phase inside the phagocytic cell. To address this question, it could be determined whether the loss of mitochondrial membrane potential is dependent on an active engulfment or degradation machinery.

2.1.4 Conclusions

Based on these observations (Fig.4 and 5), it can be concluded that in both situations, physiological and *egl-1*-induced cell death, an early hallmark is the remodeling of the normal tubular mitochondria into a more fragmented state. Thus, *C. elegans* can be added to a growing list of organisms, in which mitochondrial fragmentation is observed in the process of cell death. Interestingly, a late event in the death process appears to be the loss of mitochondrial membrane potential. This finding indicates that during physiological cell death, mitochondria are modulated during two distinct phases of the death process and in two distinct ways, during an early step, consisting of fragmentation, and during a late step, consisting of loss of mitochondrial membrane potential. The latter step likely occurs after and as a consequence of the activation of the caspase CED-3. These findings do not determine yet whether mitochondrial fragmentation is an actively regulated process during cell death as well whether it is required.

2.2 Genetic requirements of mitochondrial fragmentation in apoptotic cells

egl-1-induced cell death resulted in mitochondrial fragmentation. To further address at what step in the cell death cascade mitochondrial fragmentation occurs (see genetic pathway, Fig.9), the P*egl-1mitogfp* transgene was expressed in mutants of the central death pathway, in which all physiological cell death events are effectively blocked. It was determined whether mitochondria in these mutants fragment upon expression of the transgene like in wild-type animals. The most interesting question was whether this mitochondrial fragmentation was dependent on the caspase CED-3.

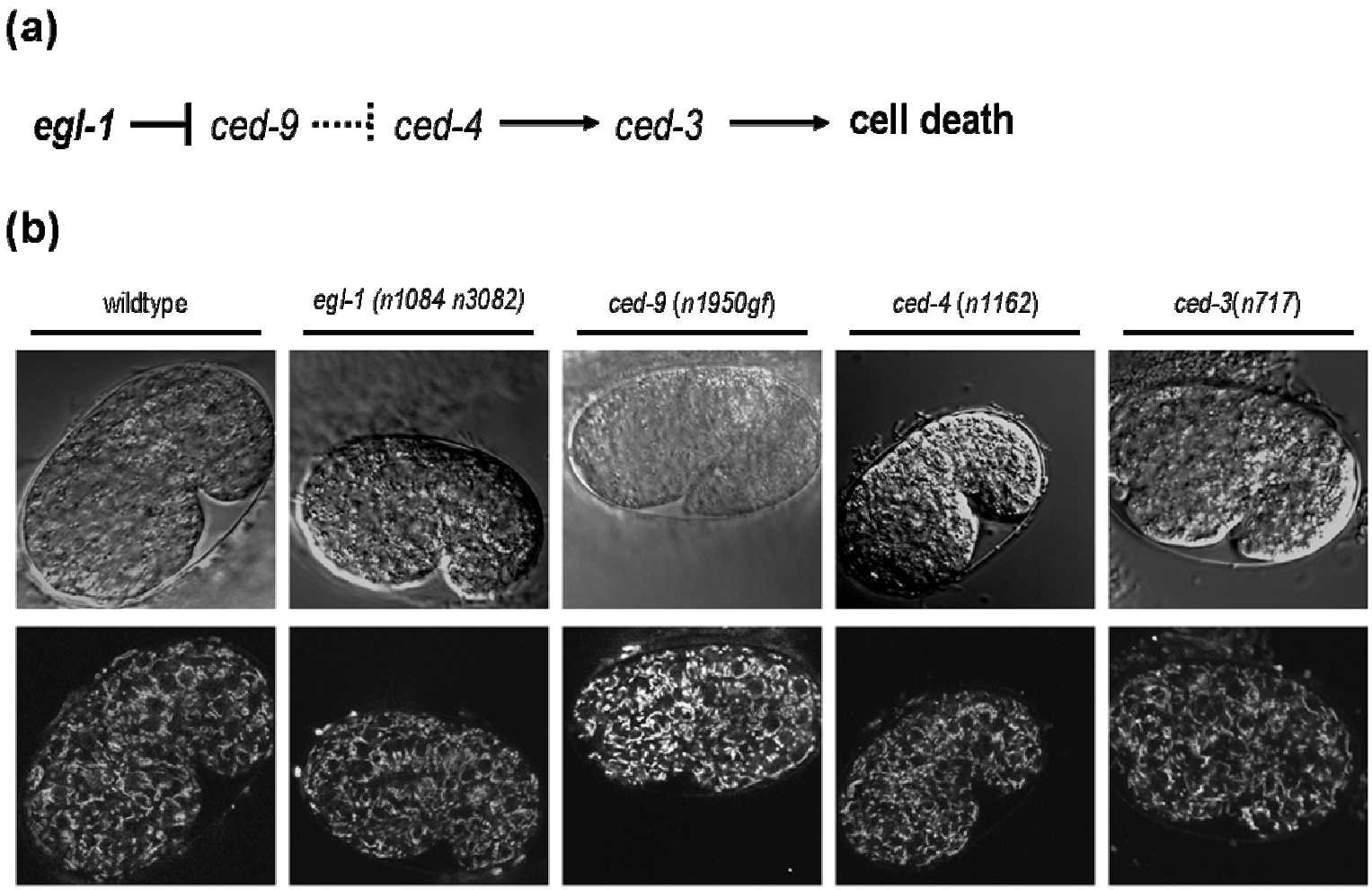

Figure 9. Mitochondrial morphology in various cell death mutants, in which most cell deaths are blocked.
(a) Genetic pathway leading to activation of cell death. Mutants defective in *egl-1*, *ced-4*, or *ced-3* or mutants with a hyper-functional *ced-9* have a block in physiological cell death. (b) *egl-1(n1084 n3082)*, *ced-9(n1950gf)*, *ced-4(n1162)*, and *ced-3(n717)* do not affect mitochondrial morphology in general. Representative confocal images of rhodamine (bottom) and DIC (top) of wild-type, *egl-1(n1084 n3082)*, *ced-9(n1950gf)*, *ced-4(n1162)*, and *ced-3(n717)* animals at the comma to 1 ½-fold stage of embryonic development. Mitochondria were stained with rhodamine B hexyl ester as described in Methods. Images of rhodamine-stained embryos represent single confocal planes.

Before mitochondrial dynamics could be examined in these mutants, it was essential to ensure that they did not display a general mitochondrial morphology defect. Therefore, mitochondrial morphology in these mutants was examined by rhodamine staining (Fig.9b). Mutants analyzed were the loss-of-function mutants *egl-1*(*n1084 n3082*), *ced-4*(*n1162*) and *ced-3*(*n717)* and the gain-of-function mutant *ced-9*(*n1950*gf). Based on rhodamine staining, all four mutants appeared to have a normal mitochondrial morphology and distribution (n≥10) (Fig.9b). This result suggested that mitochondria in these mutants are respiring and not severely compromised functionally, that they maintain a potential, and that they are not significantly abnormal in size or shape. However, even though rhodamine staining appears normal, rhodamine staining only reflects the state of the mitochondrial matrix. The mutants could potentially have abnormal mitochondria at the ultra-structural level, such as aberrant outer membranes or abnormal cristae.

2.2.1 Genetic requirements of mitochondrial fragmentation in cells destined to die

Since mitochondrial morphology appeared overall normal in the cell death mutants, it could be asked whether the same dynamic changes in mitochondrial morphology observed in apoptotic cells of wild-type animals occurred in the corresponding cells in the various mutants. In order to examine this, wild-type animals carrying the integrated P_{egl-1}*mitogfp* array were crossed into the different *ced* mutant backgrounds. “Cells

destined to die" were then imaged in the mutants for at least 25 min from the appearance of mitoGFP. Within 25 min after mitoGFP appearance, dying cells normally die and turn into a refractile corpse in wild-type animals (Fig. 6).

In *egl-1*(*n1084 n3082*) mutants, in which no functional EGL-1 protein is made, mitochondrial fragmentation was not observed over an extended period of time (Fig.10). When examining the merged mitoGFP/rhodamine picture (Fig.10, third panel from the top), the area indicative of a cell expressing the transgene, and consequently representing a cell destined to die, can be seen in white. Throughout recording, this cell maintains a mitochondrial structure indistinguishable from that in neighboring cells seen in purple. This tubular and connected morphology was maintained for 19 min 31 s from the time of initial appearance of GFP. As mentioned above, 25 min would have been sufficient to cause the formation of a corpse in the case of cells undergoing cell death in wild-type animals. (Fig.6). Thus, it could be concluded that mitochondrial fragmentation does not occur in cells destined to die without *egl-1* activity.

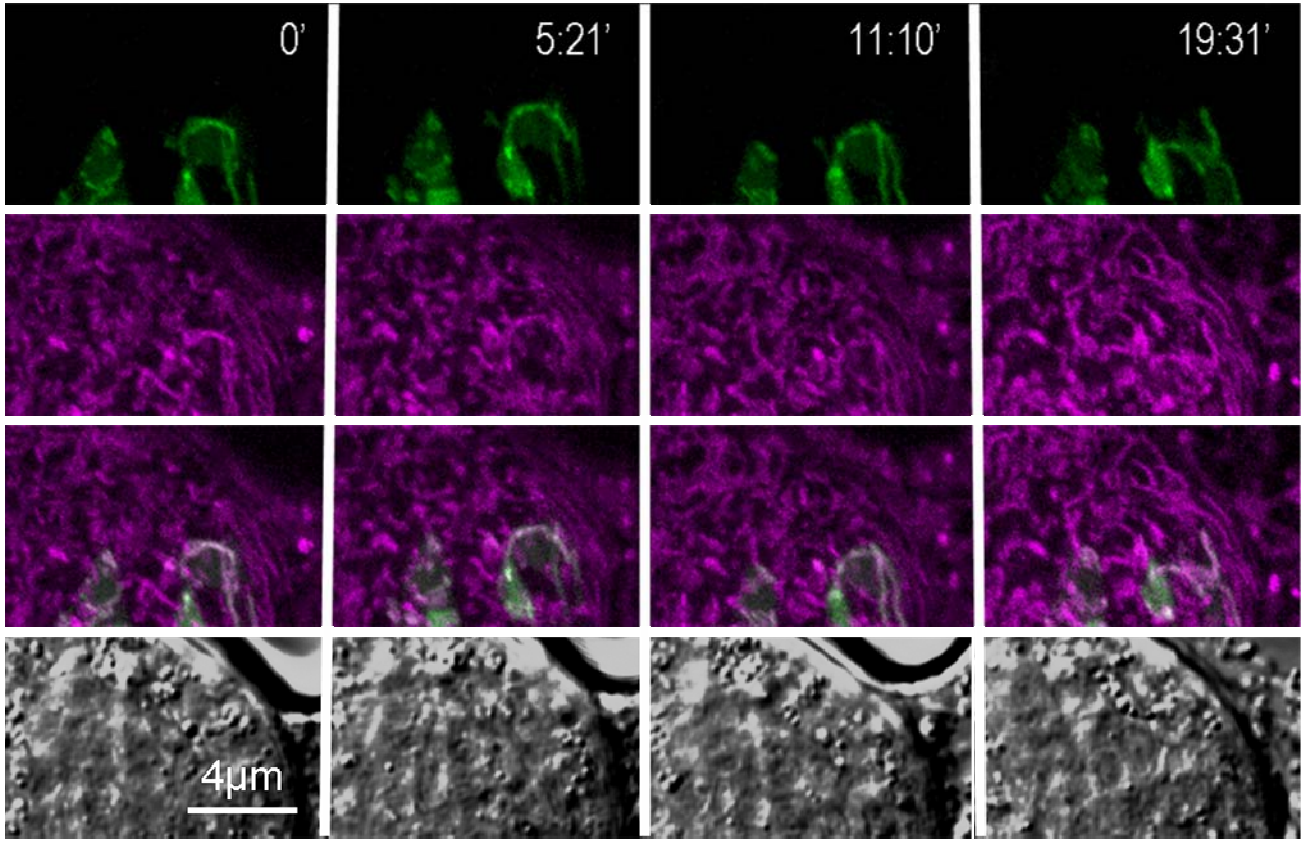

Figure 10 . Mitochondrial morphology in a cell destined to undergo programmed cell death in the *egl-1(n1084 n3082)* mutant.
Representative time-lapse series of confocal mitoGFP, rhodamine, mitoGFP/rhodamine overlay and DIC images (from top to bottom) of *egl-1(n1084 n3082)* animal carrying a stable P_{egl-1}*mitogfp* transgene (*bcIs49*). Images represent maximum intensity projections from four consecutive confocal image planes (0.5 µm). Scale bar, 4 µm.

EGL-1 is the most upstream component of the programmed cell death pathway in *C. elegans* (see genetic pathway, Fig.9a.). To further unravel whether mitochondrial fragmentation occurs upstream or downstream of other core cell death components, imaging was performed in the various mutant backgrounds. In summary (Fig.11, 12 and 13), in cells destined to die, fragmentation of the mitochondria was not seen in *ced-9*(*n1950*gf) (n=3) animals but was observed in *ced-4*(*n1162*) (n=2) and *ced-3*(*n717*) (n=2) animals. In cells destined to die in *ced-9*(*n1950*gf) mutants, like in *egl-1*(*n1084 n3082*) animals, mitochondria maintained a tubular web-like structure over the course of imaging (19 min 2 s). In contrast, in both *ced-4*(*n1162*) and *ced-3*(*n717*) animals, the mitochondria in cells destined to die fragmented within a short period of time. This result suggests that mitochondrial fragmentation is initiated by and dependent on *egl-1* activation and blocked by the *ced-9* gain-of-function mutation *n1950*. Also, the observed fragmentation was independent of *ced-3* and *ced-4*. Therefore, the mitochondrial fragmentation event occurs upstream of or in parallel to the activation of the caspase CED-3.

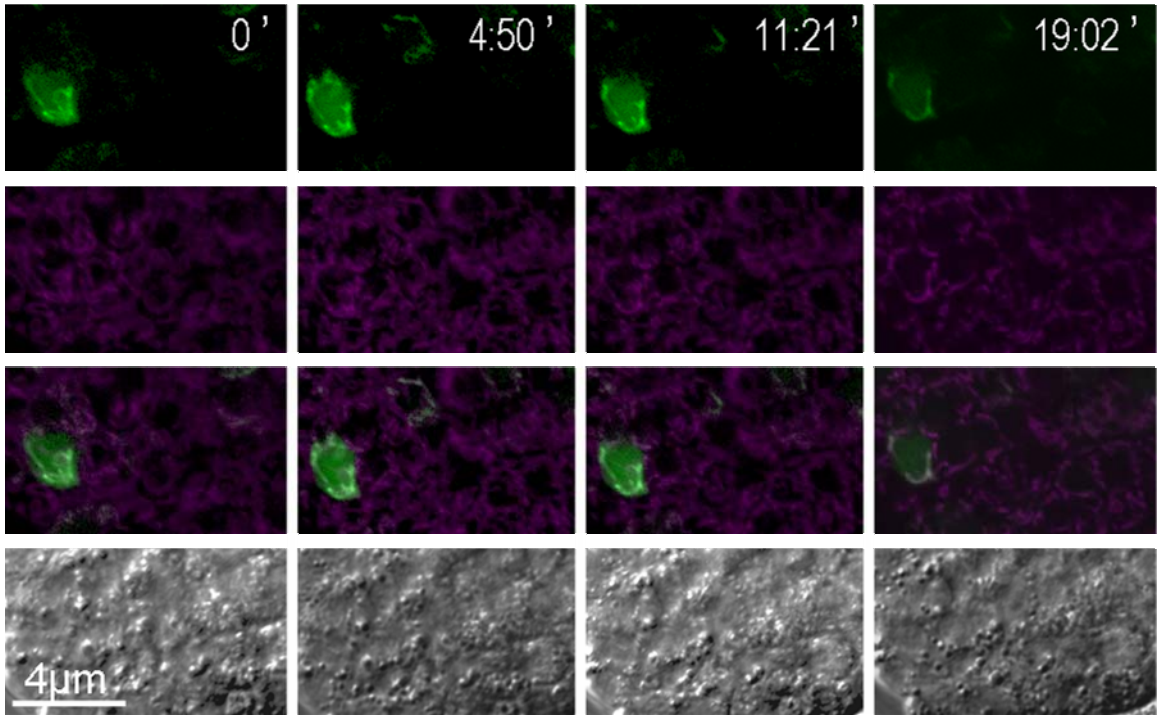

Figure 11 . Mitochondrial morphology in a cell destined to undergo programmed cell death in the *ced-9(n1950*gf) mutant.
Representative time-lapse series of confocal mitoGFP, rhodamine, mitoGFP/rhodamine overlay and DIC images (from top to bottom) of *ced-9(n1950*gf) animal carrying a stable $P_{egl\text{-}1}$*mitogfp* transgene (*bcIs49*). Images represent maximum intensity projections from four consecutive confocal image planes (0.5 µm). Scale bar, 4 µm.

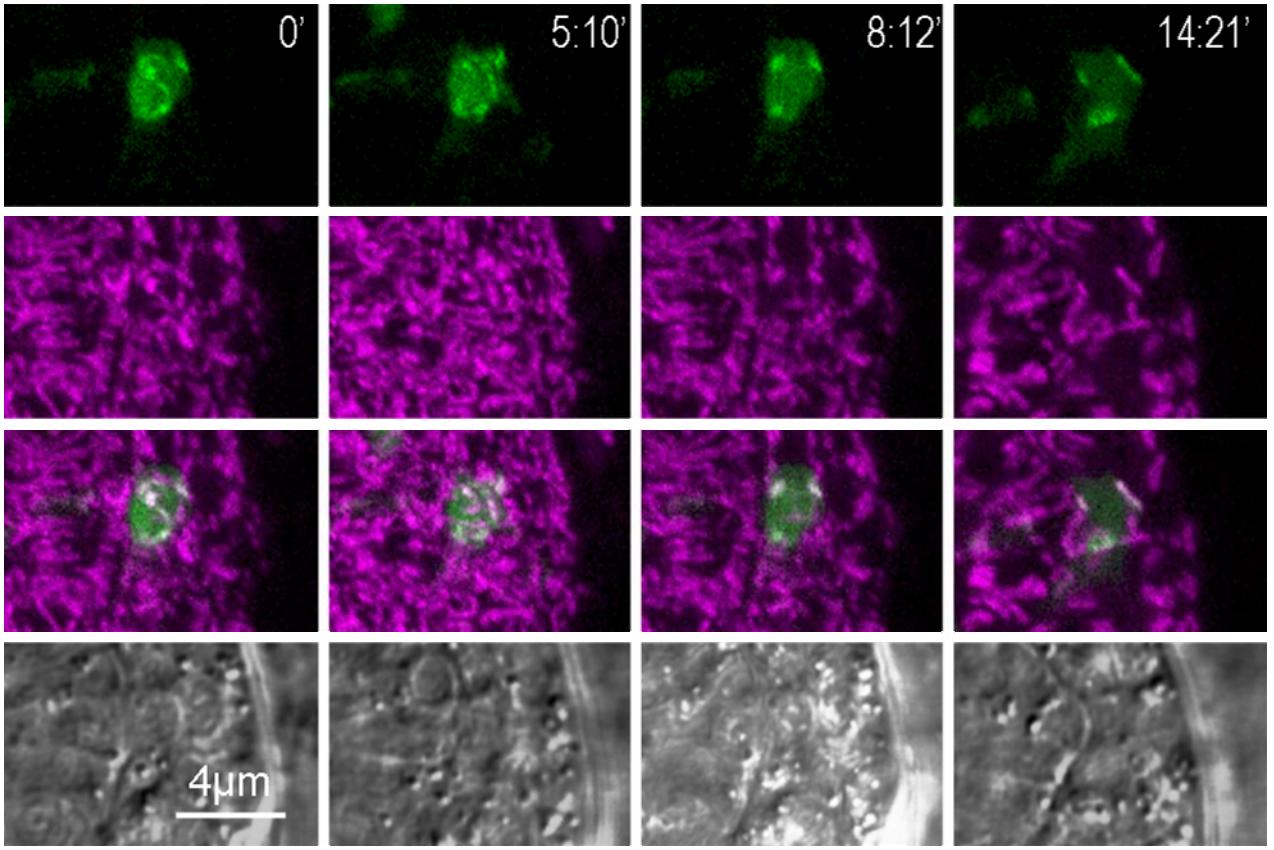

Figure 12. Mitochondrial morphology in a cell destined to undergo programmed cell death in the *ced-4(n1162)* mutant.
Representative time-lapse series of confocal mitoGFP, rhodamine, mitoGFP/rhodamine overlay and DIC images (from top to bottom) of a *ced-4(n1162)* animal carrying a stable P_{egl-1}*mitogfp* transgene (*bcIs49*). Images represent maximum intensity projections from four consecutive confocal image planes (0.5 µm). Scale bar, 4 µm.

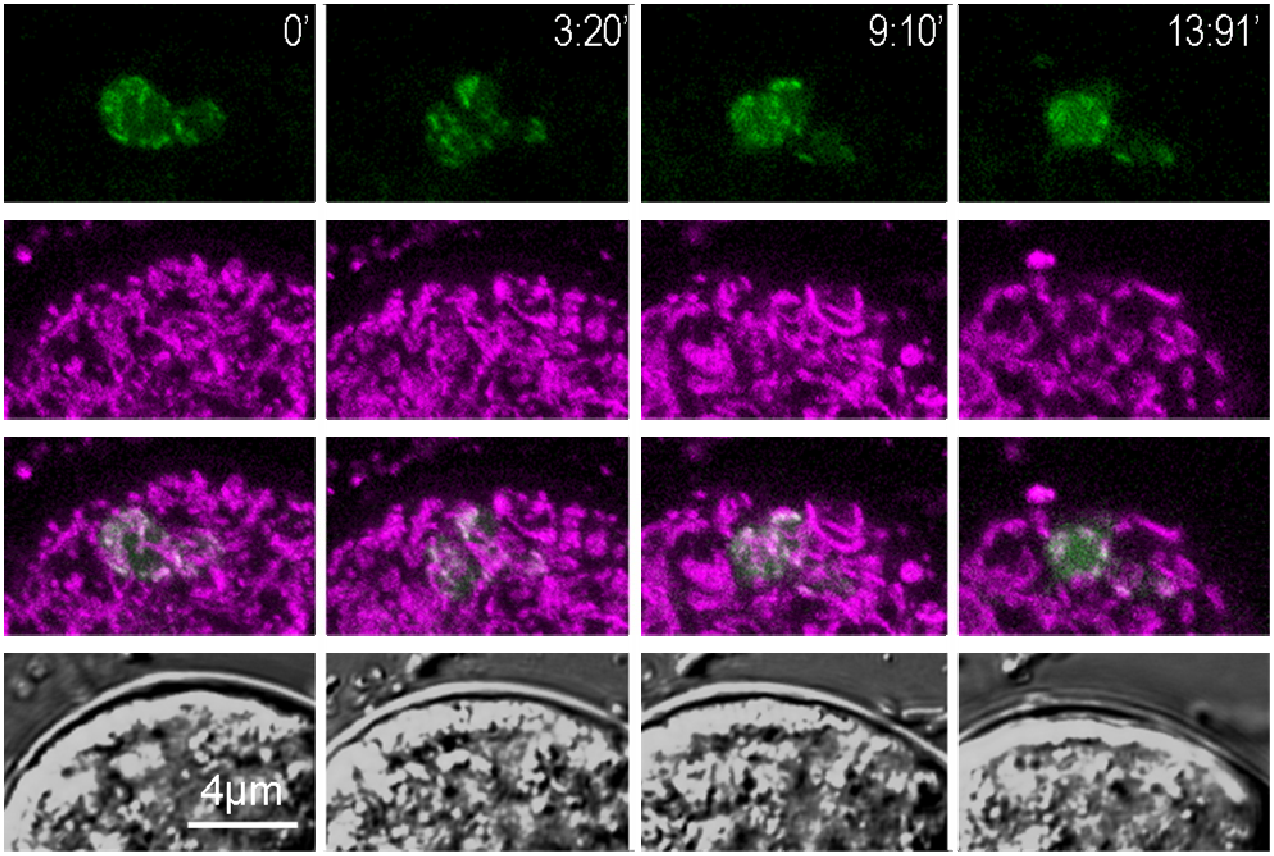

Figure 13. Mitochondrial morphology in a cell destined to undergo programmed cell death in the *ced-3(n717)* animal.
Representative time-lapse series of confocal mitoGFP, rhodamine, mitoGFP/rhodamine overlay and DIC images (from top to bottom) of *ced-3(n717)* animal carrying a stable $P_{egl\text{-}1}$*mitogfp* transgene (*bcIs49*). Images represent maximum intensity projections from four consecutive confocal image planes (0.5 µm). Scale bar, 4 µm.

In cell death mutants, most of the cells destined to die persist throughout the nematode's life (Trent et al., 1983; Ellis and Horvitz, 1986; Ellis et al., 1991). These persisting cells are termed "undead cells". Frequently, these undead cells differentiate into functional cells, such as neurons, based on morphological markers, neurotransmitter content or specific synaptic contacts sites (Avery and Horvitz, 1987; Ellis et al., 1991). For example, in *ced-3* mutants, the undead M4 sister cell differentiates into an extra M4 neuron (Avery and Horvitz, 1987). Most extra cells are likely functional and must therefore have functional mitochondria. Therefore, I was interested in determining what happens to mitochondria in undead cells Is the fragmentation that occurs early during the cell death process an irreversible event or can mitochondria 'refuse' and recover their tubular mitochondrial network?

It has been observed that under certain circumstances, such as in animals with a slightly compromised cell death pathway, cells destined to die initiate the cell death process and start to become refractile, however fail to complete the process and instead survive and differentiate (Hoeppner et al., 2001; Reddien et al., 2001). This phenomenon can also be observed at a certain low frequency in animals expressing the transgene $P_{egl\text{-}1}$*mitogfp*. In the anterior pharynx of animals expressing P*egl-1mitogfp*, there was a 30 percent chance of seeing at least one of the 16 undead cells that could potentially persist (Table 2b) ("Extra cells" counted by Barbara Conradt). Imaging of such events revealed that mitochondria initially fragment in these cells as described above and as is typical for apoptotic cells. However, the mitochondria subsequently acquired their normal tubular structure again (Fig.14).

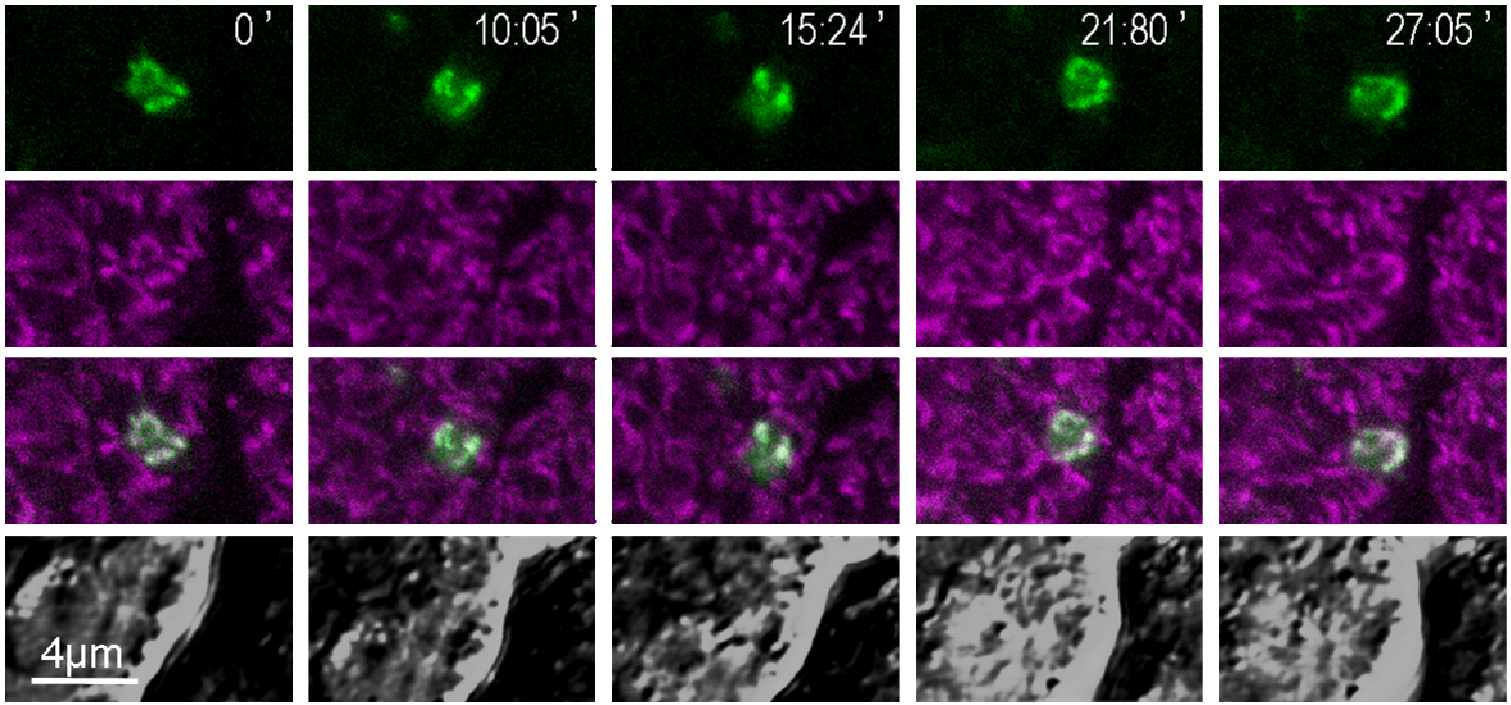

Figure 14. Mitochondrial morphology in cells initiating but not completing the cell death process.
Representative time-lapse series of confocal mitoGFP, rhodamine, mitoGFP/rhodamine overlay and DIC images (from top to bottom) of wild-type animal carrying a stable $P_{egl\text{-}1}$*mitogfp* transgene (*bcIs49*). Images represent maximum intensity projections from four consecutive confocal image planes (0.5 µm). Scale bar, 4 µm.

Similar mitochondrial morphology changes, from a tubular structure to a fragmented structure and back to a tubular structure, were observed in cells destined to die in mutants, in which cell death is slightly delayed and partially blocked due to a partial loss of function of the caspase CED-3 (Fig.15). After the appearance of GFP marking the onset of physiological cell death, mitochondria in cells destined to die in *ced-3(2427)* animals appeared as tubular structures (0 min). Shortly after, the mitochondria appeared extremely fragmented (15 min 33 s). In contrast to physiological cell death in wild-type animals, this fragmentation was not accompanied with subsequent death and the appearance of a cell corpse. Instead, the mitochondria subsequently regained their initial morphology and appeared indistinguishable from neighboring cells not expressing the transgene and therefore not destined to die (23 min 07s). In light of these observations (Fig.14 and 15), it was concluded that mitochondrial fragmentation accompanied with physiological cell death is reversible.

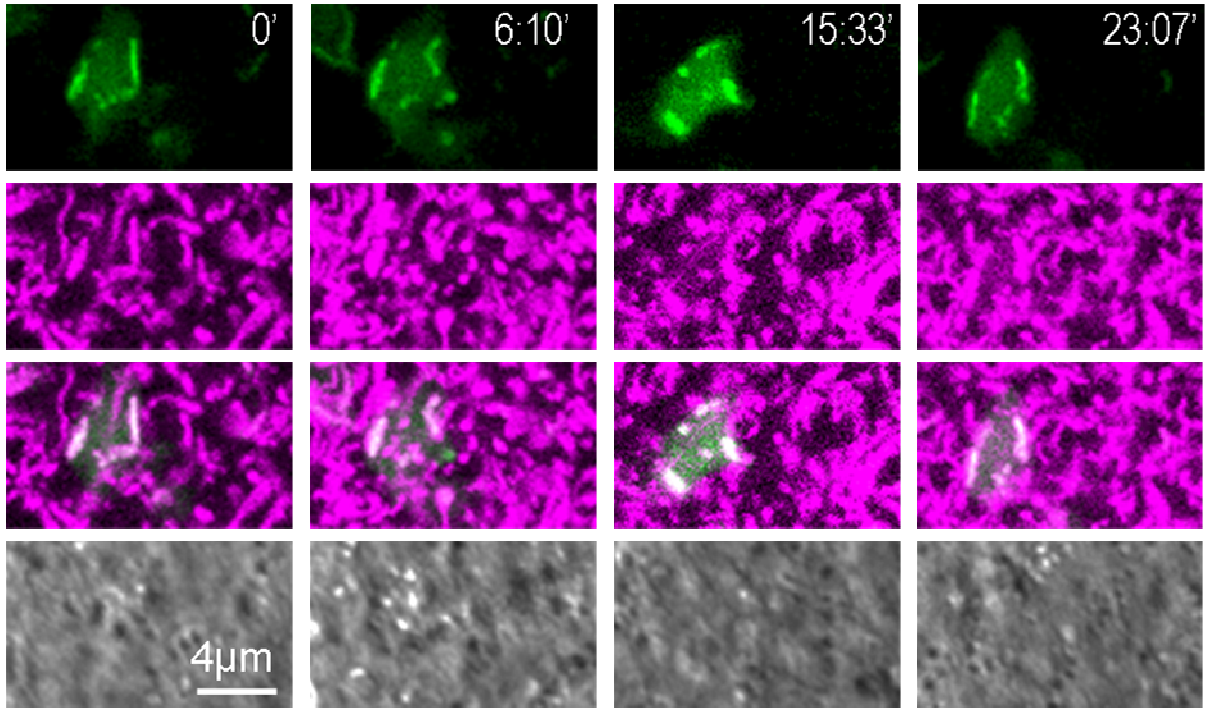

Figure 15. Mitochondrial morphology in cells undergoing programmed cell death in C. elegans but which fail to die as a consequence of the mutation ced-3(2427).
Representative time-lapse series of confocal mitoGFP, rhodamine, mitoGFP/rhodamine overlay and DIC images (from top to bottom) of ced-3(2427) animal carrying a stable Pegl-1mitogfp transgene (bcIs49). Images represent maximum intensity projections from four consecutive confocal image planes (0.5 µm). Scale bars, 4 µm.

2.2.2 Genetic requirement of mitochondrial fragmentation in cells induced to die by *egl-1* expression

It was previously demonstrated (Section 2.1.1) that ectopic *egl-1* expression under the control of a heat-inducible promoter (P_{hs}*egl-1*) induced mitochondrial changes reminiscent of the mitochondrial fragmentation observed in cells normally destined to undergo programmed cell death (Section 2.1.2). Therefore, the transgenes P_{hs}*egl-1* and P_{hs}*mitoGFP* were expressed in the various mutants (see Section 2.2) defective in cell death components (see genetic pathway, Fig.9a). In wild-type embryos, expression of *egl-1* leads to a massive induction of cell death, which has previously been shown to be blocked by the mutations *ced-9*(*n1950*gf), *ced-4*(*n1162*) and *ced-3*(*n717*) (Conradt and Horvitz, 1998). Therefore, I determined whether these mutations blocked mitochondrial fragmentation observed upon *egl-1* expression as well.

Upon the simultaneous expression of both transgenes, P_{hs}*egl-1* and P_{hs}*mitoGFP*, wild-type embryos contain a large number of cell corpses, indicative of ectopic cell death (data not shown). This reconfirmed previous reports (Conradt and Horvitz, 1998) and suggested that the expression and subsequent import of mitoGFP into the mitochondria did not significantly impede the *egl-1*-induced cell death. *egl-1* expression alone led to mitochondrial fragmentation, but not the expression of the *mitoGFP* alone (Fig.16, 2nd and 1st panel from the left, respectively). Mitochondrial fragmentation is likely mediated through EGL-1 at the mitochondria (Section 2.6.2). These results indicate that the

mitochondrial fragmentation is dependent on the BH3-only protein EGL-1, but can EGL-1 alone cause mitochondria to fragment? To address this question, the transgenes P_{hs}*egl-1* and P_{hs}*mitoGFP* were co-expressed in the various mutant backgrounds (see genetic pathway, Fig.9a). In animals lacking a functional Apaf-1-like CED-4 protein (*ced-4*(*n1162*)) or a functional CED-3 caspase (*ced-3*(*n717*)), or in animals producing a hyper-active Bcl-2-like CED-9 protein (*ced-9*(*n1950*gf)), most egl-1-induced cell death was blocked (data not shown).

In *ced-9*(*n1950*gf) embryos, *egl-1*-induced mitochondrial fragmentation was not observed, which indicates that the Bcl-2 protein CED-9 may function in the regulation of egl-*1*-induced mitochondrial fragmentation (Fig.16, 3rd panel from the left). In contrast, the loss-of-function mutations in *ced-4* or *ced-3* had no effect on *egl-1*-induced mitochondrial fragmentation (Fig.16, 4th and 5th panel from the left respectively). It was concluded that mitochondrial fragmentation occurs independently of the Apaf-1-like protein CED-4 and the caspase CED-3 (Fig.4). This suggests that mitochondrial fragmentation occurs after EGL-1 induction (through EGL-1 transcription and presumably its translocation to the mitochondria where it is thought to interact with CED-9) but before or concurrently with the release of CED-4 and the activation of CED-3 (Section 2.6.2). This finding assigns a previously unknown role to both EGL-1 and CED-9 in regulation mitochondrial morphology changes during physiological cell death in *C. elegans*.

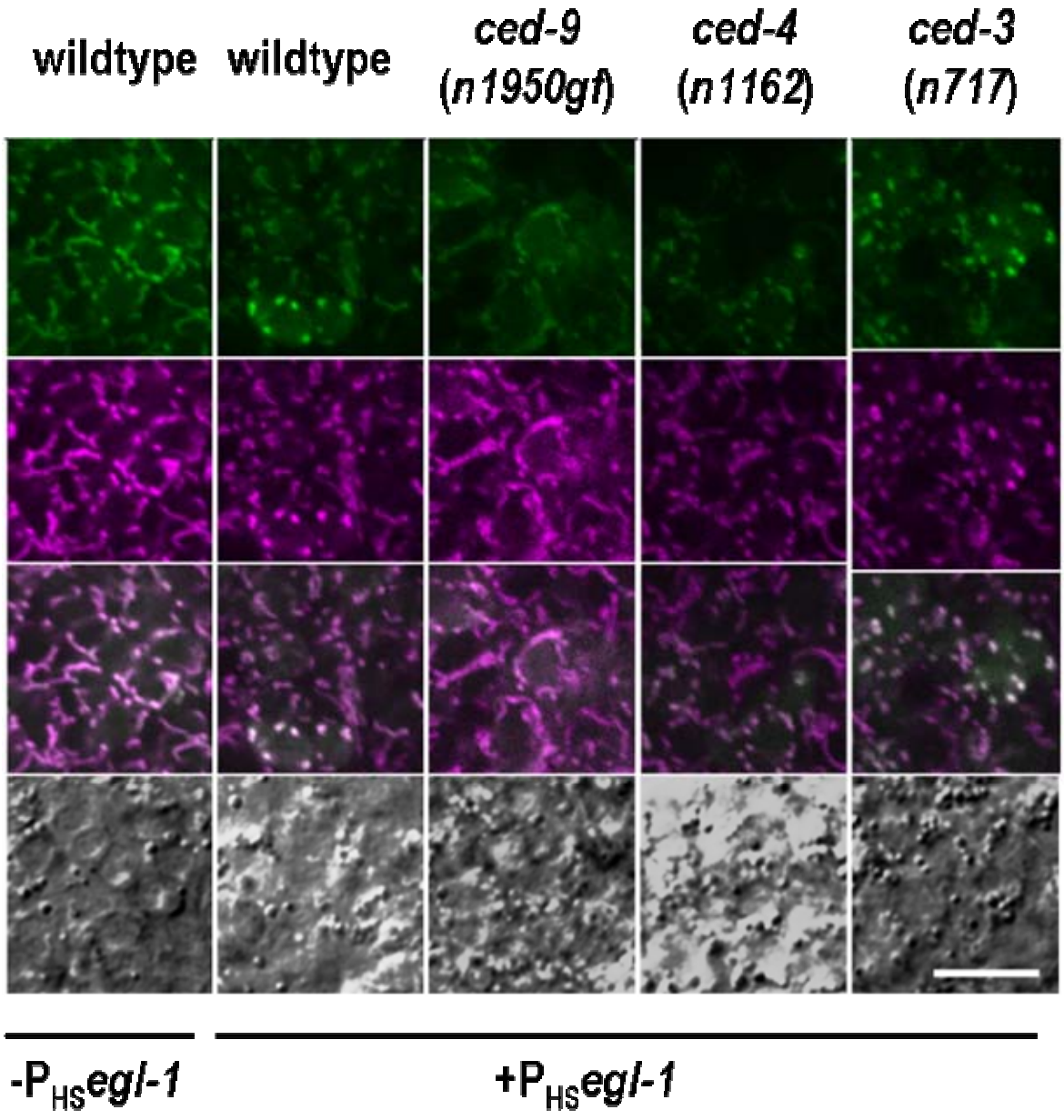

Figure 16. Mitochondrial morphology in cells induced to undergo programmed cell death by *egl-1* expression..
Representative confocal mitoGFP, rhodamine, mitoGFP/rhodamine overlay and DIC images (from top to bottom) of wild-type, *ced-9(n1950gf)*, *ced-4(n1162)* and *ced-3(n717)* animals carrying a P_{hs}*mitoGFP* transgene alone (wild-type, control, left column) or in combination with a P_{hs}*egl-1* transgene (all other columns). After induction of the transgenes, embryos were imaged at the comma to 1½-fold stage of embryonic development. Images represent single confocal image planes. Scale bar, 8 μm.

2.2.3 Conclusions

Mitochondrial fragmentation is an event that occurs upstream of *ced-3* and *ced-4* but downstream of *egl-1* and *ced-9*. EGL-1 induction and its translocation to mitochondria most likely triggers this fragmentation event. In *ced-9*(*n1950gf*) animals, which have been shown to have a single missense mutation in the *ced-9* gene resulting in a glycine-to-glutamate substitution (CED-9(G169E)) (Hengartner and Horvitz, 1994b), mitochondrial fragmentation was blocked both during physiological- and egl-1-induced cell death. Therefore, it is likely that EGL-1 must interact with a functional CED-9 protein to induce mitochondrial fragmentation in dying cells. It has previously been reported, that the *ced-9*(*n1950gf*) mutation blocks the release of CED-4 from the mitochondria (Chen et al., 2000). Could mitochondrial fragmentation require the release of CED-4 from the ternary complex of CED-9/CED-4/EGL-1 at the mitochondria? Could this be the only requirement for the EGL-1/CED-9-dependent mitochondrial fragmentation?

Since mitochondrial fragmentation appears early in the cell death process, it is now important to address the question whether it is an important step during programmed cell death. Is mitochondrial fragmentation required for cell death to proceed? As mentioned and reviewed in previous sections (Section 1.8), mitochondrial fragmentation could be due to a stimulation of mitochondria fission or an inhibition of mitochondrial fusion. Therefore, the next question that I addressed was whether blocking mitochondrial fragmentation during apoptosis could block programmed cell death events in *C. elegans*.

2.3 Blocking mitochondrial fission in apoptotic cells

2.3.1 Blocking *drp-1* function using RNA-mediated interference

One of the central components of mitochondrial fission is Dnm1p/DRP1 (reviewed in Section 1.7). Therefore, I determined whether this protein and hence mitochondrial fission has a function in programmed cell death in *C. elegans*. In a first assessment of the function of *C. elegans* DRP-1, the activity of the *drp-1* gene was reduced using RNA-mediated interference (RNAi). It had previously been reported that injecting dsRNA specific to the *drp-1* gene into adult worms led to early embryonic lethality before the 100 cell stage (Labrousse et al., 1999). This is a developmental stage prior to the occurrence of most physiological cell death events. For this reason, injecting *drp-1* dsRNA could not be used to measure effects on programmed cell death. To bypass this early embryonic lethality, I introduced *drp-1* dsRNA into worms by feeding, which leads to a less effective reduction in gene expression. Using wild-type animals or *rrf-3(pk1426)* animals (*rrf-3(pk1426)* animals have been shown to be hypersensitive to RNAi (Simmer et al., 2002; Simmer et al., 2003)), this treatment resulted in 43.2% and 21.7% embryonic lethality, respectively. This was a significant reduction in lethality from the complete embryonic arrest reported after injecting dsRNA (Labrousse et al., 1999). Since wild-type animals appeared to be more affected by ingestion of *drp-1* dsRNA than the *rrf-3* animals, cell corpses were counted in *ced-1(e1735)*; drp-1(RNAi) but otherwise wild-

type animals. (The mutation *ced-1(e1735)* blocks engulfment and therefore causes an accumulation of cell corpses (Zhou et al., 2001)). It was observed that reducing the activity of *drp-1* caused a slight but significant reduction in the accumulation in cell corpses (Fig. 17). This result suggested that drp-1(RNAi) causes either a partial block or delay in programmed cell death. I then asked whether reducing the activity of *drp-1* causes a block in cell death by scoring the number of "extra cells" in the anterior pharynx of drp-1(RNAi) animals. No "extra cells" in the anterior pharynx were observed suggesting that partially blocking *drp-1* function by RNAi did not block cell death ("Extra cells" counted by Barbara Conradt).

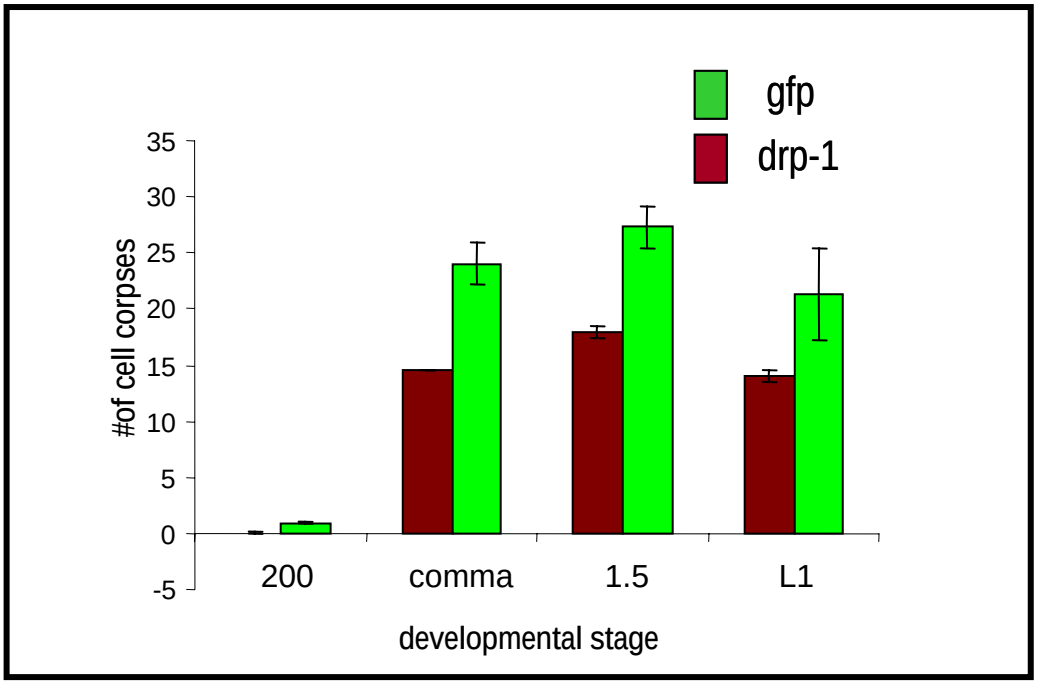

Figure 17. Delayed appearance of programmed cell deaths during embryogenesis
ced-1(e1735) animals were fed with bacteria expressing worm *drp-1* double-stranded RNA (green bars) or bacteria expressing double stranded *gfp* (dark red) and their progeny was analyzed (see Methods). The y axis represents the average number of corpses visible in the heads of embryos. Stages of embryos examined correspond to developmental stage on the x axis: 200 cell stage (200); bean and comma stage embryos (comma); 1.5-fold (1.5); and early L1 larvae with four cells in the gonad (L1). Error bars indicate standard error of the means. At least 20 embryos of each stage were examined. Experiment was performed in conditions where it was unclear to the experimenter what animal were counted, blind.

2.3.2 Isolation of a deletion in the *drp-1* gene

To further address the role of *drp-1* in *C. elegans* programmed cell death, a *drp-1* deletion was isolated from a C. elegans knock-out library. The mutation *bc259* is a deletion of 370 bp (bp1099-1468 of cosmid T12E12) within the *drp-1* locus. *bc259* eliminates parts of exons 2 and 3 (Fig.18a), resulting in a frame shift and a stop codon 66 base pairs after the 3' end of the deletion. Consequently, *bc259* results in the formation of a truncated DRP-1 protein composed of 207 amino acids of the wild-type protein and a 21 amino acid C-terminal extension in a different reading frame. Since the GTPase domain of DRP-1 is found at the N terminus, it remains completely intact however the last 505 amino acids are lost, including the dynamin-like central region, the GTPase effector domain, the pleckstrin homology domain and the Src homology domain. Therefore, it was concluded that *bc259* allele is likely a null mutation.

After isolating the deletion, animals homozygous for *drp-1(bc259)* appeared sterile (n=13). Therefore, *bc259* was crossed with the strain *dpy-13(e184sd) unc-8(n491sd)* in order to maintain the *drp-1* allele heterozygous in *trans* to the *dpy-13(e184sd) unc-8(n491sd)* chromosome. (*dpy-13(e184sd)* is semidominant i.e. animals heterozygous for this mutation have a slightly Dpy phenotype.) If *bc259* causes sterility, the strain *drp-1(bc259)/ dpy-13(e184sd) unc-8(n491sd)* is expected to generate progeny of the following phenotypes: 25 % severely dumpy and uncoordinated animals (genotype *dpy-13(e184sd) unc-8(n491sd)*), 50% slightly dumpy animals (genotype *drp-1(bc259)/dpy-*

13(e184sd) unc-8(n491sd)), and 25% non-dumpy sterile animals (genotype *drp-1(bc259)*). Non-dumpy sterile progeny was observed however at a lower than expected frequency (10-15%). Therefore, I determined whether *drp-1(bc259)/ dpy-13(e184sd) unc-8(n491sd)* animals generate embryos that arrest development during embryogenesis and therefore are not viable. I found that 10-15% of the embryos laid by *drp-1(bc259)/ dpy-13(e184sd) unc-8(n491sd)* animals arrested and never hatched, suggesting that *bc259* can cause embryonic lethality. From these results I concluded that about half of the *drp-1(bc259)* animals die during embryogenesis and that the remaining animals develop into sterile adults. The fact that about half of the *drp-1(bc259)* animals develop into sterile adults most likely is a consequence of maternal rescue.

To further resolve what is causing the lethality observed, early stage embryos were examined prior to terminal differentiation. Based on rhodamine staining, early stage wild-type embryos appeared to have tubular and evenly distributed mitochondria in all cells of the embryo (Fig.2b, Panels 1 and 2 from the left). Some of the embryos isolated from the uterus of *drp-1(bc259)/ dpy-13(e184sd) unc-8(n491sd)* hermaphrodites displayed an aberrant mitochondrial morphology. The mitochondria in these embryos appeared highly interconnected and unevenly distributed among the cells of the animals (Fig. 2b, panels 3 and 4 from the left). I concluded that these embryos most likely are homozygous for *drp-1(bc259)* and that, most probably due to a loss of mitochondrial division, mitochondria in these animals were not evenly inherited during cell division. Since mitochondrial morphology appeared defective in very early embryos, I concluded that this defect in mitochondrial division was causing the embryonic lethality. Presumably mitochondria

were not segregating properly to daughter cells, resulting in some cells that lack sufficient mitochondria to maintain certain mitochondrial function, such as energy production in the form of ATP and Ca^{2+} buffering. Before and after embryonic arrest, embryos displaying an aberrant mitochondrial morphology had refractile, corpse-like "structures" and vacuolated cells, characteristic of necrotic deaths (Fig.18b, panel 6 from the left). It had previously been reported that blocking the function of the caspase CED-3 protected embryos from lethality upon *drp-1* RNAi (Labrousse et al., 1999). Therefore, I tested whether *drp-1*(*bc259*)-induced embryonic lethality was blocked by the mutation *ced-4*(*n1162*), which prevents the activation of CED-3. I observed the mitochondrial segregation defect, embryonic lethality, cell corpse accumulation and necrotic bodies in *ced-4*(*n1162*); *drp-1*(*bc259*) embryos (Fig. 18b). For this reason, I concluded that the embryonic lethality observed was the cause of a mitochondrial segregation defect. Of interest, the accumulation of refractile structures was not blocked by *ced-4*(*n1162*), suggesting that disturbing mitochondrial function can cause cells to die in a manner that is independent of the apoptotic cell death machinery.

As mentioned above, due to maternal rescue, about half of the homozygous *drp-1*(*bc259*) embryos escaped embryonic arrest and developed into sterile adults. It was therefore possible to determine whether these "escapers" had a defect in programmed cell death. *drp-1*(*bc259*) L4 larvae did not have any extra cells in the anterior pharynx ("Extra cells" counted by Barbara Conradt). This would suggest that *drp-1* is not required for programmed cell death or, alternatively, that sufficient maternal DRP-1 protein is transferred to *drp-1*(*bc259*) embryos to fulfill its requirements in programmed cell death

during embryogenesis. I concluded that it was not possible to convincingly determine through the analysis of *drp-1(bc259)* whether or not there is an effect of a block in mitochondrial fragmentation on programmed cell death.

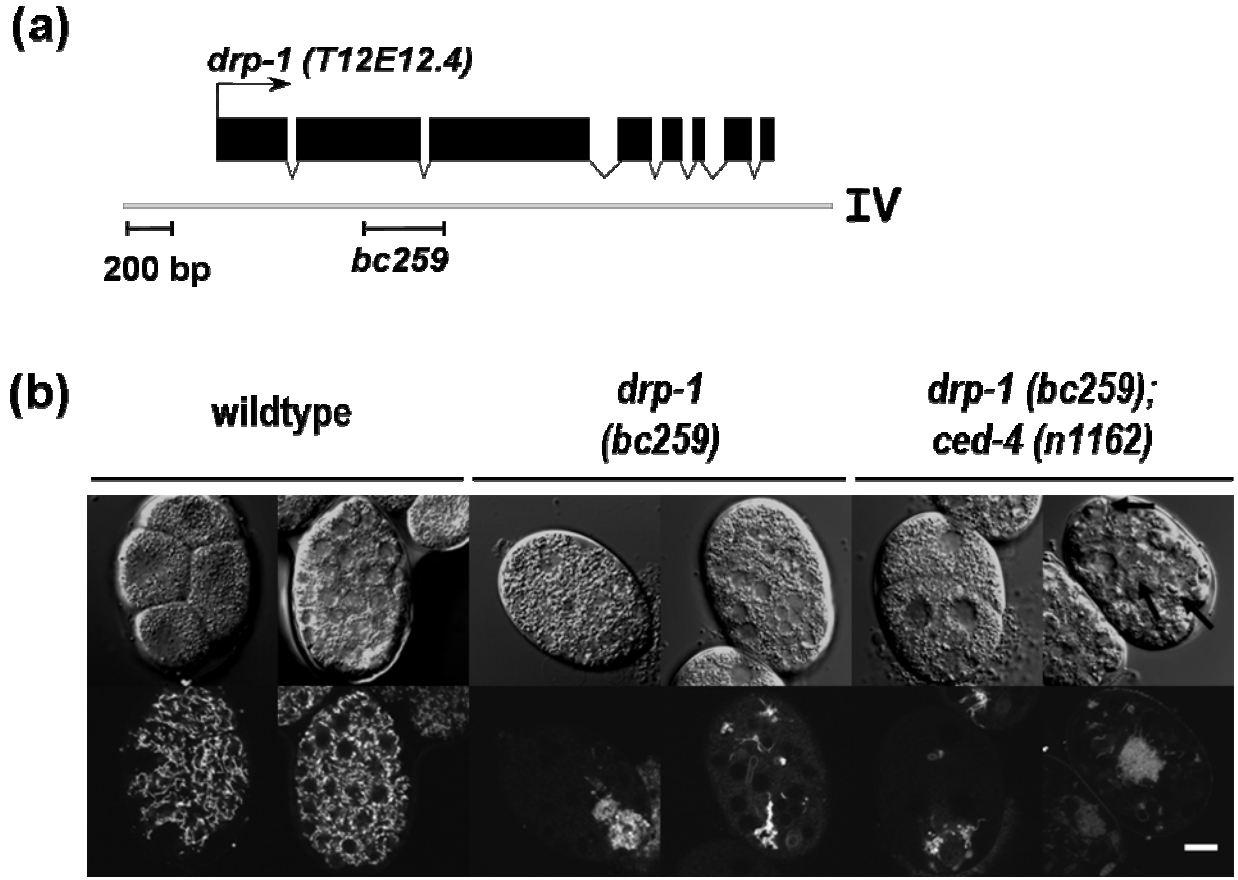

Figure 18. The effects of the *drp-1(bc259)* allele.
(a) Representation of the *drp-1* locus and the location of the bc259 deletion. The T12E12 cosmid carries drp-1(T12E12.4). The black boxes and connector lines indicate exons and introns of the *drp-1* gene, respectively. Regions deleted in bc259 are indicated under the summary of exon/intron structure and the gray bar repesents chromosome 4. (b) Mitochondrial morphology in *drp-1(bc259)* animals. DIC and rhodamine images (top and bottom panels, respectively) of wild-type animals (+/+) at the four-cell stage and at mid-gastrulation, and of arresting *drp-1(bc259)* and ced-4(n1162); *drp-1(bc259)* animals. Animals were stained as described in Methods. Images represent single confocal planes. *drp-1(bc259)*-induced embryonic lethality was not blocked by ced-4(n1162). Notice that the *drp-1(bc259)*-induced appearance of refractile structure also was not suppressed by ced-4(n1162). Black arrows point to refractile structure.

2.3.3 Transiently blocking mitochondrial fission using *drp-1(K40A)*

Since both drp-1(RNAi) and drp-1(bc259) cause embryonic lethality and consequently could not be used for the analysis of defects in programmed cell death, I sought to transiently block mitochondrial fission. The expression of a dominantly-interfering *drp-1* construct, *drp-1*(K40A), had been previously shown to efficiently block mitochondrial fragmentation in cultured mammalian cells and in *C. elegans* muscle cells (Smirnova et al., 1998; Labrousse et al., 1999). Therefore through the expression of *drp-1*(K40A), it became feasible to block mitochondrial fission in *C. elegans* transiently and not throughout the whole course of development. This approach could possibly bypass the embryonic arrest caused by *drp-1* inactivation through RNAi or *bc259*. In order to investigate the consequences of blocking mitochondrial fragmentation on programmed cell death in *C. elegans*, *drp-1*(K40A) was expressed under the control of two different promoters, the heat-inducible promoter (P_{hs}*drp-1*(K40A)) or the *egl-1* promoter (P_{egl-1}*drp-1*(K40A)). As shown in Figure 19, the expression of *drp-1*(K40A) in embryos indeed efficiently blocked mitochondrial fragmentation. In embryos expressing *mitoGFP* alone, mitochondria appeared tubular and evenly distributed (Fig.19, left column). Expression of both *drp-1*(K40A) and *mitoGFP* led to mitochondrial clumping, the appearance of mitochondria of irregular shape and size, and a loss of the even, tubular distribution of mitochondria seen in wild-type embryos (Fig.19, right column). Thus the expression of *drp-1*(K40A) effectively decreased the rate of mitochondrial fission in embryos and led to a more fused mitochondrial phenotype.

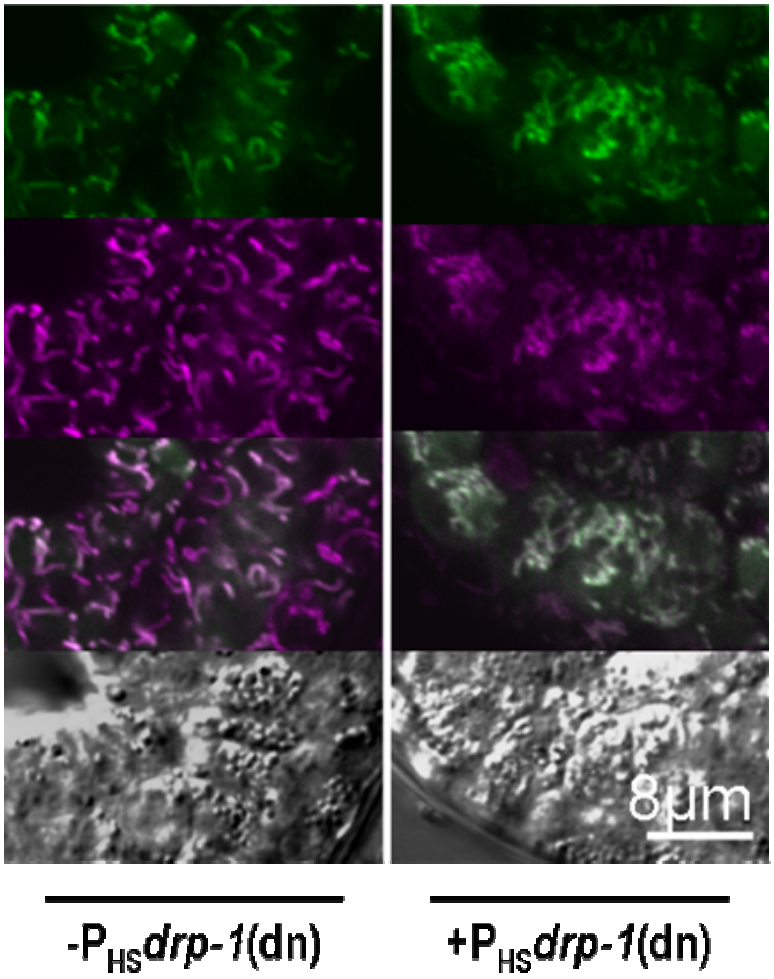

Figure 19. Overexpressing *drp-1*(K40A) phenocopies the effect *drp-1(bc259)*.
Representative confocal mitoGFP, rhodamine, mitoGFP/rhodamine overlay and DIC images (from top to bottom) of wild-type animals carrying a P_{hs}*mitogfp* transgene alone (control, left column), or in combination with a P_{hs}*drp-1*(K40A) transgene (*drp-1*(K40A), right column). After induction of the transgenes, embryos were imaged at the comma to 1½-fold stage of embryonic development. Images represent single confocal image plane. Scale bar, 8 µm.

Like the terminal arrest phenotype seen in *drp-1(bc265)* homozygous embryos, the expression of *drp*-1(K40A) under the control of the heat-inducible promoters led to14-30 percent embryonic lethality (Table 1). Fortunately, more than 70 percent of embryos expressing the transgene developed into adults. Furthermore, the percent lethality could be significantly reduced, if the heat shock was applied later during embryogenesis i.e. to embryos that had been laid rather than embryos in utero. It had previously been reported that blocking programmed cell death protected embryos from embryonic lethality upon *drp-1* RNAi (Labrousse et al., 1999). Therefore, I determined whether the mutations *ced-9*(*n1950*gf) and *ced-4* (*n1162*), which block most physiological cell death, could suppress the embryonic lethality induced by expression of the *drp*-1(K40A) transgene. The percentage of transgenic embryos that arrested upon *drp-1*(K40A) expression was not significantly reduced by these two cell death mutations (Table 1). Therefore I concluded that blocking programmed cell death does not suppress the lethality induced by impeding drp-1-induced mitochondrial fission.

Table 1.

The expression of *drp-1*(K40A) under the control of a *C. elegans* heat shock promoter causes lethality, which is not suppressed by *ced-9*(*n1950gf*) or *ced-4*(*n1162*).

		% embryonic lethality			
Line	% transgenic animals	no heat shock	n	heat shock	n
Control – 1	58	0	50	3	240
Control – 2	47	0	92	3	91
P_{hs} *drp-1*(K40A) – 1	33	4	70	14	73
P_{hs} *drp-1*(K40A) – 2	60	8	52	28	53
P_{hs} *drp-1*(K40A) – 3	48	6	51	17	48
P_{hs} *drp-1*(K40A) – 4; *ced-9*(*n1950gf*)	55	2	41	20	49
P_{hs} *drp-1*(K40A) – 5; *ced-4*(*n1162*)	58	10	67	25	71

P_{hs} *drp-1*(K40A) – 6; *ced-4*(*n1162*)	58	5	38	32	69

Table 1. Expression of *drp-1*(K40A) causes embryonic lethality.

Animals carrying extrachromosomal arrays of the P_{hs} *drp-1*(K40A) transgene or control extrachromosomal arrays were subjected to heat shock during embryogenesis as described in the material and methods ("heat shock") or were not subjected to heat shock ("no heat shock"). The percent transgenic animals were determined by counting the total number of adults and the number of nonUnc transgenic adults that developed from the treated embryos. The percent embryonic lethality was determined by counting the total number of embryos treated and the number of embryos that failed to hatch within 24hr of the treatment. The complete genotypes of the animals were (from top to bottom): *unc-76*(*e911*); P_{hs}*mitogfp* lines 1, 2, *unc-76*(*e911*); P_{hs}*mitogfp* + P_{hs} *drp-1*(K40A) lines 1, 2, 3, *ced-9*(*n1950*gf); *unc-76*(*e911*); P_{hs}*mitogfp* + P_{hs} *drp-1*(K40A) line 4, *ced-4* (*n1162*); *unc-76*(*e911*); P_{hs}*mitogfp* + P_{hs} *drp-1*(K40A) lines 4,5

I could now address whether temporally reducing mitochondrial fission could block cell death by determining whether inducing the expression of *drp-1*(K40A) within embryos could lead to the accumulation of extra cells in the anterior pharynx. During the development of the anterior pharynx, 16 cells undergo programmed cell death (Sulston and Horvitz, 1977; Sulston et al., 1983). Strong loss-of-function mutations in pro-apoptotic genes, such as *egl-1*, *ced-4*, or *ced-3,* lead to the persistence of 11-12 of these 16 cells in the anterior pharynx (Yuan and Horvitz, 1990; Yuan and Horvitz, 1992; Conradt and Horvitz, 1998). Weak loss-of-function mutations in *egl-1*, *ced-4*, or *ced-3* lead to the survival of 2-3 of these 16 cells (Reddien et al., 2001). Expression of *drp-1*(K40A) from the heat-inducible promoter P_{hs}*drp-1*(K40A) in embryos led to the appearance of about 2-3 extra cells in the anterior pharynx in 4 of 4 transgenic lines created ("Extra cells" counted by Barbara Conradt) (Table 2a). Without the heat shock treatment during embryogenesis, 0.5-1 extra cells were present in the anterior pharynx of transgenic animals, suggesting that the heat-inducible promoters may be slightly leaky (Table 2A). The heat-induced, *drp-1*(K40A)-dependent effect on programmed cell death observed is comparable to the effect caused by weak loss-of-function mutations in the pro-apoptotic genes. A similar result was obtained when *drp-1*(K40A) was expressed

under the control of the *egl-1* promoter (P_{egl-1}*drp-1*(K40A)) (Table 2B). In 5 of 7 transgenic lines generated, disruption of mitochondrial fission led to the persistence of 1-3 extra cells in the anterior pharynx. From these results, I concluded that programmed cell death can be partially inhibited by reducing DRP-1-mediated fission of mitochondria.

Table 2.

A. The expression of *drp-1*(K40A) under the control of a heat-inducible promoter blocks programmed cell death

	- heat shock			+ heat shock		
Line	average number of extra cells	± SD	range	average number of extra cells	± SD	Range
control – 1	0.1	0.3	0-1	0.2	0.2	0-1
control – 2	0	0	0	0.1	0.1	0-1
control – 3	0.1	0.3	0-1	0.1	0.1	0-1
P_{hs} *drp-1*(K40A) - 1	0.4	0.7	0-2	2.9	1.5	1-5
P_{hs} *drp-1*(K40A) - 2	0.5	0.8	0-2	2.2	1.7	0-6
P_{hs} *drp-1*(K40A) - 3	0.9	1.5	0-5	2.8	1.9	0-7
P_{hs} *drp-1*(K40A) - 4	1.0	1.0	0-3	2.7	1.4	0-4

B. The expression of *drp-1*(K40A) under the control of the *egl-1* promoter blocks programmed cell death

Line	average number of extra cells	± SD	range
control - 1	0.3	0.5	0-1
control - 2	0.3	0.5	0-1
control - 3	0.1	0.4	0-1
control - 4	0.1	0.4	0-1
control - 5	0.1	0.4	0-1
control - 6	0.2	0.4	0-1
control - 7	0.1	0.3	0-1
P_{egl-1} *drp-1*(K40A) - 1	0.4	0.7	0-2
P_{egl-1} *drp-1*(K40A) - 2	2.3	1.1	1-4
P_{egl-1} *drp-1*(K40A) - 3	0.3	0.5	0-1

P_{egl-1} *drp-1*(K40A) - 4	3.1	1.7	1-7
P_{egl-1} *drp-1*(K40A) - 5	3.4	1.2	1-6
P_{egl-1} *drp-1*(K40A) - 6	2.8	1.2	0-5
P_{egl-1} *drp-1*(K40A) - 7	1.1	1.4	0-3

Table 2. Expression of *drp-1*(K40A) blocks programmed cell death.

(a) Transgenes were activated and the number of extra cells in the anterior pharynx determined as described in Methods. ±SD, standard deviation. Number of animals counted was n=10-15. The complete genotypes of the animals were (from top to bottom): *unc-76*(*e911*); P_{hs}*mitogfp* lines 1, 2, 3, *unc-76*(*e911*); P_{hs}*mitogfp* + P_{hs} *drp-1*(K40A) lines 1, 2, 3, 4. (b) The number of extra cells in the anterior pharynx was determined as described in Methods. ±SD, standard deviation. The complete genotypes of the animals were (from top to bottom): *unc-76*(*e911*); P_{egl-1}mitogfp lines 1-7, *unc-76*(*e911*); P $_{egl-1}$*drp-1*(K40A) + P_{egl-1}mitogfp lines 1-7. Analysis was performed by Barbara Conradt.

2.3.4 Conclusion

I addressed whether a block in mitochondrial fragmentation has any effect on programmed cell death by using *drp-1*(RNAi) or the drp-1 deletion bc259. However, *drp-1*(RNAi) and *drp-1(bc259)* both caused embryonic arrest and subsequent lethality. Specifically, affected embryos arrested at a developmental stage prior to the stage, during which most programmed cell death events occur during embryogenesis. Therefore, using these two approaches, it was impossible to determine whether a block in mitochondrial fragmentation could affect programmed cell death. The embryonic arrest was likely due to a defect in mitochondrial inheritance, since it was apparent that the mitochondria did not segregate properly upon cell division thereby disrupting cellular homeostasis.

Fortunately, using both temporally- and spatially-inducible systems to block mitochondrial fission, I could effectively determine the effects that impeding fission has on programmed cell death. Allowing freshly plated worms to lay eggs for up to an hour before inducing the expression of *drp-1*(K40A) allowed a significant proportion of

embryos expressing the transgene to proceed through development. Since the cells, whose inappropriate survival is analyzed using the anterior pharynx assay, are normally destined to die at around 425 min after the first cleavage of the zygote, a significant proportion of the embryos would be expressing the *drp-1*(K40A) transgene at that time. Using the spatially-inducible system, the *drp-1*(K40A) transgene is expressed in cells destined to die only at the time of the initiation of programmed cell death. Both promoters allowed a bypass of the lethality and maternal effect obstacle observed in *drp-1(bc259)* animals. In both systems, a block in mitochondrial fragmentation induced by reducing *drp-1* function partially blocked programmed cell death; I concluded that mitochondrial fragmentation is at least partially required for cell death.

However, the inhibitory effect of *drp-1*(K40A) expression on programmed cell death could be regarded as rather weak, since using the two different promoters only about 2 to 3 of the possible 16 cells in the anterior pharynx inappropriately survived. For the following two reasons it is conceivable that this effect in principle could be significantly stronger than what was actually observed: 1) The *drp-1*(K40A) transgenes were expressed by generating extra chromosomal arrays through DNA injections into the hermaphrodite gonad. Transgenic animals generated using this method are considered mosaic since cells spontaneously lose the extrachromosomal arrays at a certain frequency during cell division. For this reason, it is almost certain that not all of the 16 cells destined to die in the anterior pharynx will contain the extrachromosomal array and therefore the transgenes. To ensure the expression of the transgene in every cell of the animals, the extrachromosomal array could be stably integrated into a *C. elegans*

chromosome, however, integration can theoretically lead to gene silencing. 2) Expression of *drp-1*(K40A) from the *egl-1* promoter could occur too late to be effective in many of the cell death events. A sufficient amount of time might be required for *drp-1*(K40A) to be expressed and for the DRP-1(K40A) protein to effectively block mitochondria fragmentation, before the initiation of cell death by the EGL-1 protein. To get around this limitation, the experiment could be repeated in a genetic background in which programmed cell death is delayed, such as in the background of a ced-8 mutation or a weak ced-3 loss-of-function mutation (Stanfield and Horvitz, 2000; Reddien et al., 2001). However, despite these technical limitations, I can conclude that blocking mitochondrial fragmentation by expressing *drp-1*(K40A) can at least partially block programmed cell death.

2.4 Is *drp-1* expression sufficient to induce cell death?

I have demonstrated that ectopic *egl-1* expression induced mitochondrial fragmentation prior to programmed cell death as detected by the appearance of cell corpses. Since the mitochondrial fragmentation is at least partially required for cell death, it was important to next address whether mitochondrial fission alone can induce programmed cell death. It was previously reported that overexpression of the wild-type *drp-1* gene, *drp-1*(wt), in muscle cells of adult *C. elegans* enhances the fission of mitochondria, resulting in fragmented organelles within cells (Labrousse et al., 1999). Therefore, the effect of overexpressing *drp-1(wt)* in embryos as opposed to muscle cells was determined. Overexpression of *drp-1*(wt) during embryogenesis using the heat-inducible promoter

(P_{hs}*drp-1*(wt)) caused mitochondria to fragment in a similar manner to what was previously seen in mitochondria in muscle cells (Fig. 20, right column). Fragmented mitochondria were apparent within minutes after the 45 minute heat shock used to induce expression of *drp-1*.

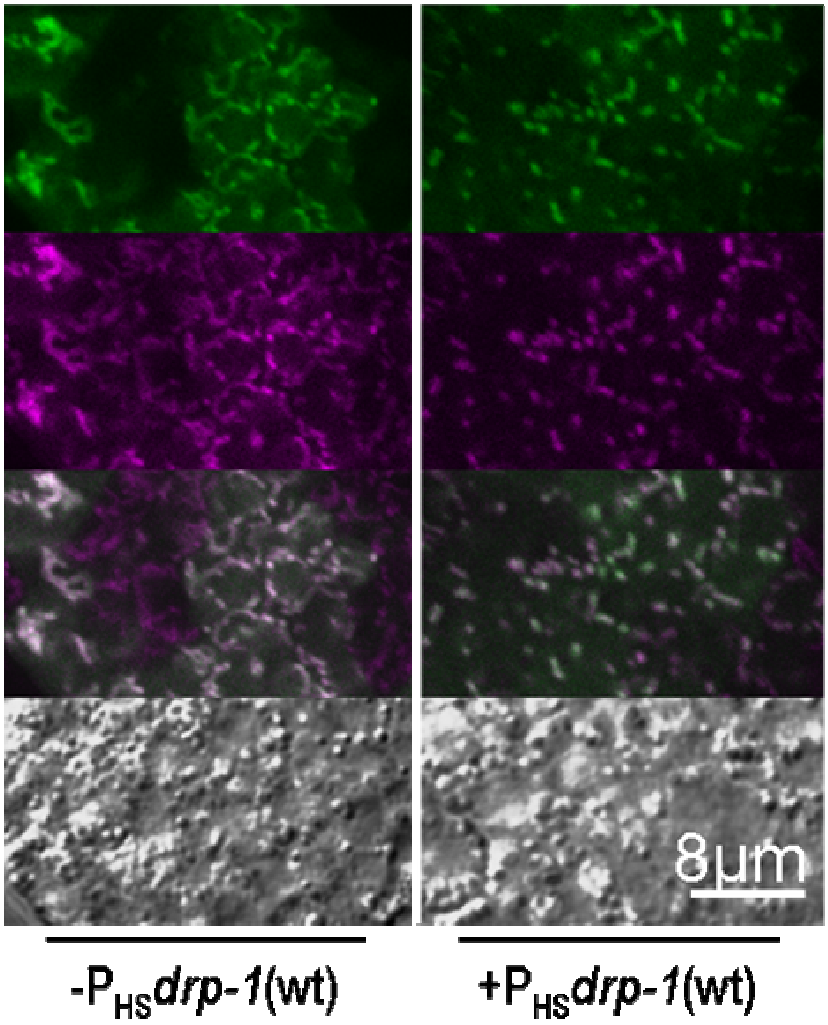

Figure. 20 Overexpressing *drp-1*(wt) causes mitochondrial fragmentation.
Representative confocal mitoGFP, rhodamine, mitoGFP/rhodamine overlay and DIC images (from top to bottom) of wild-type animals carrying a P$_{hs}$*mitogfp* transgene alone (control, left column), in combination with a P$_{hs}$*drp-1*(wt) transgene (*drp-1*(wt), right column). After induction of the transgenes, embryos were imaged at the comma to 1½-fold stage of embryonic development. Images represent single confocal image plane. Scale bar, 8 µm.

2.4.1 Cell death induction upon expression of *drp-1*

Surprisingly, within an hour after the initial 45 min heat shock and after mitochondrial fragmentation could be observed, the number of refractile corpses in embryos overexpressing drp-1 increased similar to what is seen after *egl-1* overexpression (Fig.21, left column, summarized in Table. 3). Furthermore, the appearance of refractile corpses in embryos overexpressing drp-1 was suppressed by the mutations *egl-1*(*n1084 n3082*), *ced-9*(*n1950*gf), *ced-4*(*n1162*) or *ced-3*(*n717*) (Fig.21, data quantified in Table 3). This observation confirmed that the *drp-1*-induced corpses represent ectopic programmed cell death.

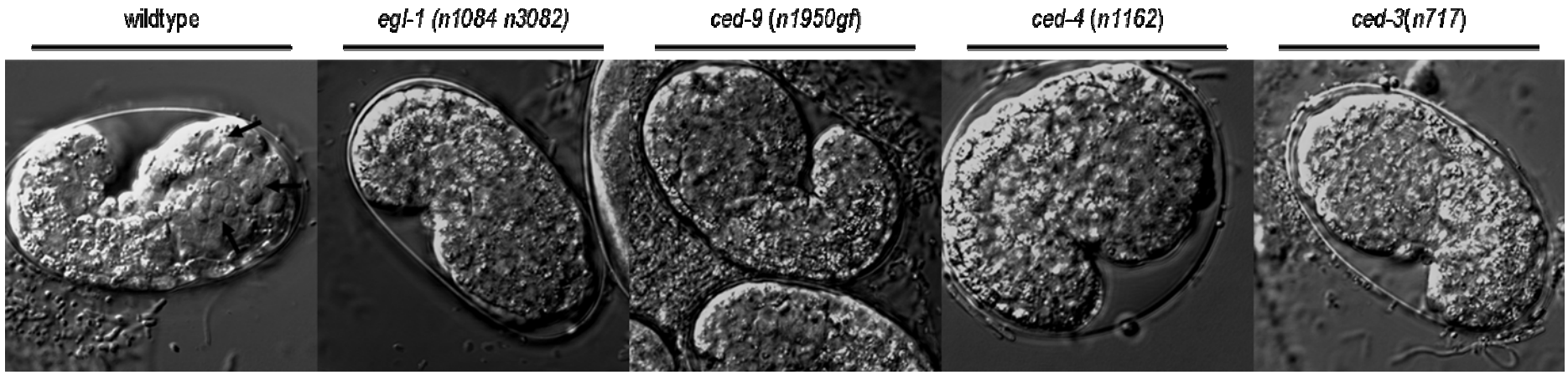

Figure 21. Overexpression of wild-type *drp-1* leads to ectopic cell corpses, which are suppressed by *ced* mutations.
Representative confocal DIC images of animals carrying a P_{hs}*mitogfp* transgene + P_{hs}*drp-1*(wt). Representative images after induction of transgene (from left to right): unc-76(e911); P_{hs}*mitogfp* + P_{hs}*drp-1*(wt),unc-76(e911) egl-1(n1084 n3082); P_{hs}*mitogfp* + P_{hs}*drp-1*(wt), ced-9(*n1950*gf); unc-76(e911); P_{hs}*mitogfp* + P_{hs}*drp-1*(wt), ced-4 (n1162); unc-76(e911); P_{hs}*mitogfp* + P_{hs}*drp-1*(wt), ced-3(n717); unc-76(e911); P_{hs}*mitogfp* + P_{hs}*drp-1*(wt). The black arrows point to a refractile corpse.

Table 3. Expression of wild-type *drp-1* causes programmed cell death

Line	average number of corpses	± SD	range	n
control – 1	7.9	0.8	7-9	12
control – 2	8.6	1.6	7-12	15
P_{hs} *drp-1*(wt) – 1	18.6	2.7	15-25	15
P_{hs} *drp-1*(wt) – 2	25.2	3.4	17-27	15
P_{hs} *drp-1*(wt) – 3	22.4	2.7	19-28	15
P_{hs} *drp-1*(wt) – 4	10.1	2.0	7-13	15
P_{hs} *drp-1*(wt) – 5	21.1	3.0	19-28	15
egl-1(*n1084 n3082*); P_{hs} *drp-1*(wt) - 6	0	0	0	7
egl-1(*n1084 n3082*); P_{hs} *drp-1*(wt) - 7	0	0	0	10
ced-9(*n1950*gf); P_{hs} *drp-1*(wt) - 8	1.1	1.0	0-3	10
ced-9(*n1950*gf); P_{hs} *drp-1*(wt) - 9	1.6	1.1	0-3	9
+/+; P_{hs} *drp-1*(wt) - 9*	15.4	3.3	10-22	13
ced-9(*n1950*gf); P_{hs} *drp-1*(wt) - 10	2.7	1.1	0-4	10
+/+; P_{hs} *drp-1*(wt) - 10*	17.9	3.9	10-22	13
ced-4(*n1162*); P_{hs} *drp-1*(wt) - 11	0	0	0	9
ced-4(*n1162*); P_{hs} *drp-1*(wt) - 12	0.1	0.3	0-1	11
ced-4(*n1162*); P_{hs} *drp-1*(wt) - 13	0.2	0.4	0-1	12
ced-4(*n1162*); P_{hs} *drp-1*(wt) - 14	0	0	0	15
ced-3(*n717*); P_{hs} *drp-1*(wt) - 15	0	0	0	20
ced-3(*n717*); P_{hs} *drp-1*(wt) - 16	0	0	0	26
ced-3(*n717*); P_{hs} *drp-1*(wt) - 17	0	0	0	6
+/+; P_{hs} *drp-1*(wt) - 17*	19.6	4.1	12-24	15
ced-3(*n717*); P_{hs} *drp-1*(wt) - 18	0	0	0	30

Table 3. Expression of wild-type *drp-1* causes programmed cell death.

Transgenes were activated and the number of refractile corpses determined as described in Methods. ±SD, standard deviation. Animals analyzed were at the comma stage of embryogenesis. The complete genotypes of the animals were (from top to bottom): *unc-76*(*e911*); P_{hs}*mitogfp* lines 1, 2, *unc-76*(*e911*); P_{hs}*mitogfp* + P_{hs}*drp-1*(wt) lines 1-5, *unc-76*(*e911*) *egl-1*(*n1084 n3082*); P_{hs}*mitogfp* + P_{hs}*drp-1*(wt) lines 6, 7, *ced-9*(*n1950*gf); *unc-76*(*e911*); P_{hs}*mitogfp* + P_{hs}*drp-1*(wt) lines 8, 9, 10, *ced-4* (*n1162*); *unc-76*(*e911*); P_{hs}*mitogfp* + P_{hs}*drp-1*(wt) lines 11-14, *ced-3*(*n717*); *unc-76*(*e911*); P_{hs}*mitogfp* + P_{hs}*drp-1*(wt) lines 15-18. Lines marked with * were crossed out of the respective *ced* backgrounds to confirm that the transgenes were active in a wild-type background.

To summarize the data shown in Table 3, the numbers of cell corpses observed in embryos overexpressing *drp-1* were determined using 1½-fold embryos that had been heat shocked 2 hours prior to being analyzed. In four out of five independent transgenic

lines generated, the overexpression of *drp-1*(wt) resulted in a large increase in the number of refractile corpses within embryos (18 to 25 cell corpses), which is significantly more than in control lines expressing *mitoGFP* alone (7-8 cell corpses). This increase in the number of cell corpses after *drp-1*(wt) expression was not seen in the background of the mutations *egl-1*(*n1084 n3082*), *ced-9*(*n1950*gf), *ced-4*(*n1162*) or *ced-3*(*n717*). This result suggests that overexpressing *drp-1*(wt) leads to the formation of corpses through the activation of the programmed cell death pathway. To confirm that the extrachromosomal arrays generated in the various mutant backgrounds were functional, some of the arrays were crossed into a wild-type background (Table 3, arrays contained in lines 9, 10, and 17) and analyzed for their ability to cause ectopic programmed cell death. All arrays tested induced an increased number of cell corpses in a wild-type background after heat shock. This result confirmed that the transgenes indeed were active and that the lack of cell corpses in the mutant background was due to the suppression of drp-1(wt)-induced cell death rather than the loss of functional arrays.

An increased number of refractile corpses at a particular stage of development, such as the comma to 1½-fold stage of development, is not unequivocal evidence that cells normally destined to live inappropriately undergo cell death. One alternative explanation for an increase in cell corpses is that the efficient clearance of cells normally destined to die is blocked, leading to an accumulation of their cell corpses. That the increased number of cell corpses detected in embryos expressing *drp-1*(wt) is not a result of a block in engulfment is supported by two separate lines of evidence: 1) The lethality caused by the overexpression of *drp-1*(wt) during embryogenesis; 2) The comparison of the number

of cell corpses accumulating in embryos overexpressing *drp-1*(wt) and embryos displaying engulfment defects.

drp-1(wt) expression during embryogenesis results in significant embryonic lethality (33-38 %) (Table 4). Unlike the lethality induced by overexpression of *drp-1*(K40A), this lethality is significantly suppressed by mutations in egl-1, ced-9, ced-4 and ced-3 (Table 4 and data not shown). For instance, in a *ced-9*(*n1950*gf) background, embryonic lethality in transgenic lines is reduced to less than 10 %. However, after crossing the array into a wild-type background, lethality was reinstated. The lethality was also partially blocked by the mutations *egl-1(n1084 n3082), ced-4(n1162)*, or *ced-3(n717)* (data not shown). This suppression of lethality in *ced* mutants suggests that the embryonic lethality was a direct consequence of increased programmed cell death. Additionally, a block in engulfment per se does not lead to embryonic lethality in *C. elegans*. Therefore, this finding supports the conclusion that the increase in cell corpses observed after drp-1(wt) expression are a consequence of ectopic cell death rather than a block in engulfment.

Table 4. Ectopic expression of *drp-1*(wt) under the control of heat-inducible promoters causes embryonic lethality, which is at least partially suppressed by *ced-9*(*n1950*gf)

	no heat shock		heat shock	
Line	**% transgenic animals (n)**	**% embryonic lethality (n)**	**% transgenic animals (n)**	**% embryonic lethality (n)**
control - 1	58 (50)	0 (50)	53 (232)	3 (240)
control - 2	47 (92)	0 (92)	38 (88)	3 (91)
P_{hs} *drp-1*(wt) - 2	69 (109)	1 (110)	41 (89)	33 (133)
P_{hs} *drp-1*(wt) - 3	69 (156)	0 (156)	54 (95)	35 (147)
P_{hs} *drp-1*(wt) - 5	76 (98)	0 (98)	52 (42)	38 (68)

ced-9(*n1950*gf); P_{hs} *drp-1*(wt) - 9	58 (66)	0 (66)	51 (111)	8 (121)
+/+; P_{hs} *drp-1*(wt) - 9*	57 (77)	0 (77)	34 (109)	32 (161)
ced-9(*n1950*gf); P_{hs} *drp-1*(wt) - 10	52 (105)	2 (107)	46 (137)	9 (150)
+/+; P_{hs} *drp-1*(wt) - 10*	59 (58)	3 (60)	31 (136)	18 (165)

Table 4. **Expression of wild-type *drp-1* causes embryonic lethality**, **suppressed by *ced-9(n1950gf)*.**
Animals carrying extrachromosomal arrays of the P_{hs} *drp-1*(wt) transgene or control extrachromosomal arrays were subjected to heat shock during embryogenesis as described in the material and methodes ("heat shock") or were not subjected to heat shock ("no heat shock"). The percent transgenic animals was determined by counting the total number of adults and the number of nonUnc transgenic adults that developed from the treated embryos. The percent embryonic lethality was determined by counting the total number of embryos treated and the number of embryos that failed to hatch within 24hr of the treatment. The complete genotype of the animals were (from top to bottom): *unc-76(e911);* P_{hs} *mitogfp* lines 1, 2, *unc-76(e911);* P_{hs} *mitogfp* + P_{hs} *drp-1*(wt) lines 2, 3, 5, *ced-9(n1950*gf*); unc-76(e911);* P_{hs} *mitogfp* + P_{hs} *drp-1*(wt) line 9 and 10. Lines marked with * were crossed out of the *ced-9*(*n1950*gf) background to confirm that the transgenes were active in a wild-type background.

The numbers of cell corpses observed in embryos overexpressing *drp-1*(wt) were determined using 1½-fold embryos that had been heat shocked 2 hours prior to analysis (Table 3). Cell corpse counts were performed at this time point after heat shock in order to eliminate measuring indirect effects of *drp-1* expression such as effects caused by mitochondrial stress. Interestingly, the average number and range of cell corpses in embryos expressing drp-1(wt) increases significantly when cell corpses were analyzed 3 to 4 hours after the initial heat shock. At this time point, 1½-fold embryos expressing *drp-1*(wt) had an average of 55.4 cell corpses with a range of 31 to 74 cell corpses (cell corpses counted by Barbara Conradt). Similarly, using the same experimental set-up, 1½-fold embryos expressing *egl-1* had an average of 49.3 cell corpses with a range of 25 to 76 cell corpses (cell corpses counted by Barbara Conradt). For comparison, wild-type 1½-fold embryos have an average of 7.3 cell corpses (range 4-10) (cell corpses counted by Barbara Conradt) and *ced-7(n1892); ced-5(n1812)* 1½-fold embryos, in which engulfment is severely defective, have an average of 43.4 cell corpses with a range of 38 to 51 (cell corpses counted by Barbara Conradt). Considering these numbers it can be convincingly ruled out that that the more than 70 corpses observed after *drp-1*-induction

are the result of a defect in engulfment. This observation strengthens the conclusion that overexpression of *drp-1*(wt) induces ectopic programmed cell death rather than a block in engulfment. Therefore I concluded that DRP-1-induced excessive mitochondrial fission causes ectopic programmed cell death.

2.4.2 Mitochondrial morphology in embryos expressing *drp-1*(wt)

I demonstrated that *drp*-1-induced mitochondrial fragmentation preceded *drp*-1-induced ectopic cell death. I then asked whether mitochondrial fragmentation is also observed in the various ced mutants upon *drp-1* expression and, more importantly, whether a functional EGL-1 protein is required for *drp*-1-induced mitochondrial fragmentation.

Since it could be observed that within an hour of the initial heat shock that induced *drp-1* overexpression mitochondria appeared highly fragmented, embryos were imaged two hours of the initial heat shock to ensure that the *drp-1* expression had enough time to take effect. Similar to *egl-1*-induced mitochondrial fragmentation, *drp-1*-induced mitochondrial fragmentation was not blocked in *ced-4*(*n1162*) or *ced-3*(*n717*) animals (Fig.21, 5th and 6th column from the left, respectively). This result suggests that *drp-1*-induced mitochondrial fragmentation, however not drp-1-induced cell death, is independent of CED-4 or CED-3. Interestingly, like *egl-1*- induced mitochondrial fragmentation, *drp-1*-induced mitochondrial fragmentation was blocked by *ced-9*(*n1950*gf) (Fig.21 4th column from the left). Unexpectedly, *egl-1*(*n1084 n3082*) failed to

block mitochondrial fragmentation after *drp-1* expression (Fig.21 3rd column from the left). Therefore, the overexpression of *drp-1*(wt) must bypass the requirement for *egl-1* function in mitochondrial fragmentation but not in the subsequent death, since *drp-1* expression did not induce ectopic cell death in *egl-1*(*n1084 n3082*) embryos. This is a very surprising result and, at first glance, seems incomprehensible for the following reasoning: *egl-1* does not appear to be expressed in cells destined to survive, however, the overexpression of *drp-1* and *drp-1*-induced mitochondrial fragmentation kills cells destined to survive. If these two results are true it is unclear how DRP-1-induced cell death can be blocked in *egl-1*(*n1084 n3082*) embryos (For discussion see section 2.4.3).

The ability of *ced-9*(*n1950*gf) to block drp-1-induced mitochondrial fragmentation under conditions that bypass EGL-1 function, strongly suggests that CED-9 might have another role in programmed cell death autonomous of its known pro-survival function.

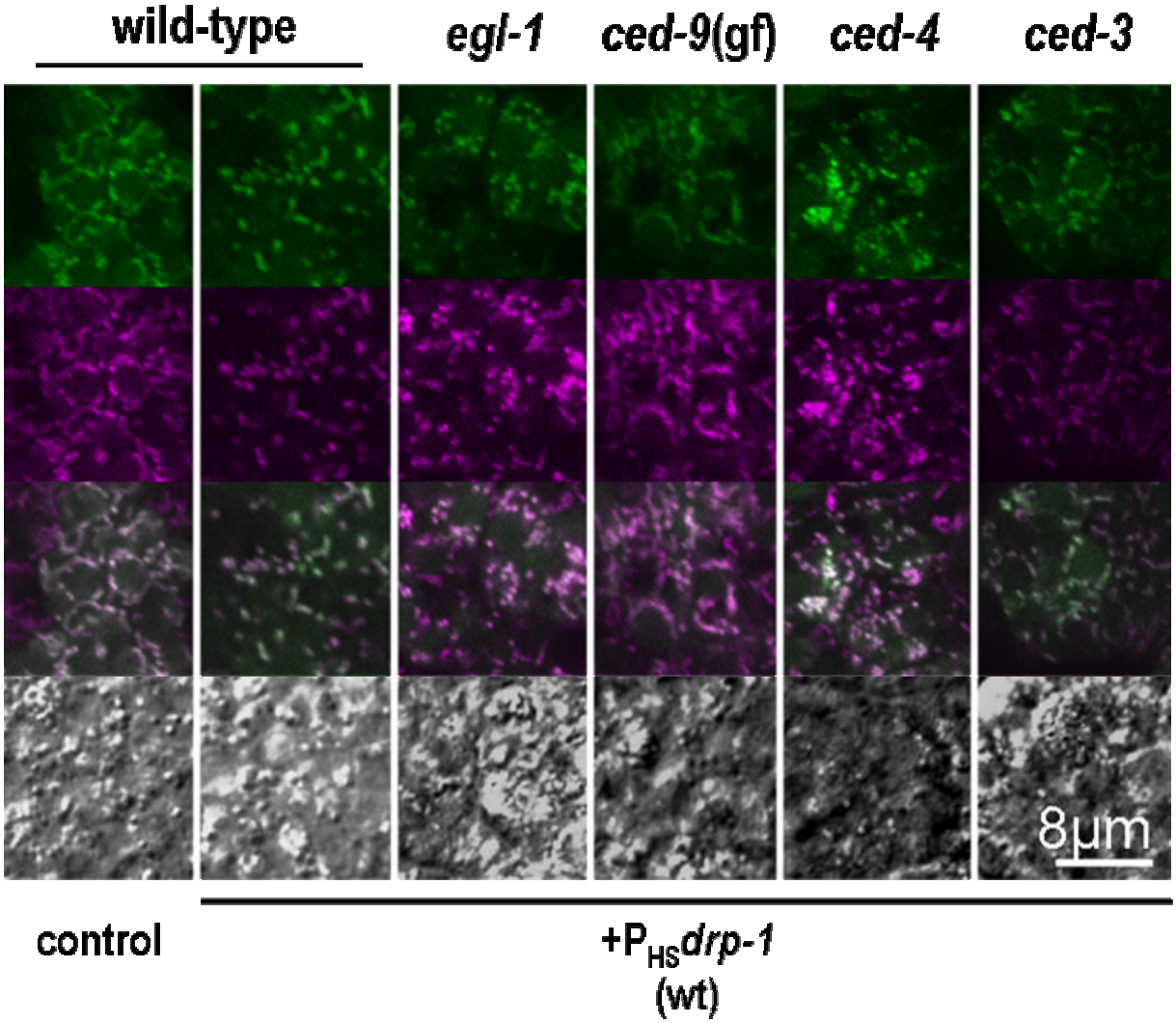

Figure 22. Mitochondrial morphology in embryos expressing *drp-1*(wt).
Representative confocal mitoGFP, rhodamine, mitoGFP/rhodamine overlay and DIC images (from top to bottom) of wild-type animals carrying a P_{hs}*mitogfp* transgene alone (control, first column), or in combination with a P_{hs}*drp-1*(wt) transgene (*drp-1*(wt), second column), and egl-1(n1084 n3082), ced-9(*n1950*gf), ced-4(n1162), or ced-3(n717) animals carrying P_{hs}*mitogfp* and P_{hs}*drp-1*(wt) (other columns, as indicated). After induction of the transgenes, embryos were imaged at the comma to 1½-fold stage of embryonic development. Images represent single confocal image planes. Scale bar, 8 µm.

2.4.3 Conclusions

By overexpressing *drp-1*(wt), three important aspects of mitochondria in *C. elegans* programmed cell death were discovered: 1) DRP-1-induced mitochondrial fragmentation precedes DRP-1-induced cell death. 2) DRP-1-induced cell death requires a functional egl-1, ced-4, and ced-3 gene, suggesting that mitochondrial fragmentation affects cell death by acting through these genes. 3) *ced-9(*gf*)* mutation inhibits DRP-1-induced mitochondrial fragmentation. These facts assign a crucial role to mitochondria in the cell-death pathway in *C. elegans*. However, as always, these observations lead to more questions than answers. A few of the important questions are: What is the function of the mitochondria and, more specifically, why are mitochondria required to fragment during the process of programmed cell death? What is the role of CED-9 during mitochondrial fragmentation and could this role represent CED-9's killing function, which has been previously defined genetically? How is it that *egl-1*, which seems to exclusively be expressed in cells destined to die, is required for the death of cells destined to survive?

How might mitochondrial fragmentation contribute to killing?

Mitochondrial fragmentation must affect cell death by acting through the genes *egl-1*, *ced-4*, and *ced-3*. The mitochondrial fragmentation pathway must therefore enhance the central cell death pathway or act as a positive feedback loop on the central pathway.

There could be several reasons why mitochondrial fragmentation is required for cell death. Since mitochondrial fission allows for several mitochondria to exist independently within a single cell, it is likely that different populations of mitochondria are required for death to proceed. A possible scenario could be that upon initiation of cell death, mitochondria fragment in order for independent mitochondria to serve some unknown apoptotic function while other mitochondria maintain their function in the generation of ATP, as apoptosis is an energy-requiring process.

What could this yet unknown apoptotic function of mitochondria be? The most probable function is the release of apoptogenic factors from the mitochondrial inter membrane space. From studies in mammals it is known that a plethora of apoptogenic substances are released from the inter membrane space of the mitochondria, including Cytochrome *c*. The release of these molecules may significantly comprise the mitochondria, requiring independent organelles to maintain cellular homeostasis and drive the apoptotic reaction. As mentioned previously, it is still unknown whether Cytochrome *c* or other apoptogenic factors are released from mitochondria during cell death in *C. elegans* and play a role in *C. elegans* physiological cell death. Assigning a role for Cytochrome *c* in cell death in *C. elegans* is hindered because there are two Cytochrome *c* genes found in the worm genome, Cytochrome *c* is essential for viability of *C. elegans* and biochemical studies are hard to perform within the embryo. Using the time-lapse system that I established, it might now be possible to test for the release of specific factors from the inter membrane space during physiological death (see section 4.3)

What is the role of CED-9 in mitochondrial fragmentation?

I demonstrated that *ced-9*(*n1950*gf) blocks *egl-1*- and *drp-1*-induced mitochondrial fragmentation. It was previously determined that *ced-9*(*n1950*gf) compromises the ability of the CED-9 protein to release CED-4 (Chen et al., 2000), and it was proposed that the n1950 mutation interferes with EGL-1 binding . Because *egl-1*(lf) does not block *drp-1*-induced mitochondrial fragmentation, the inability of DRP-1 to induce mitochondrial fragmentation in *ced-9*(*n1950*gf)'s embryos is not a result of the inability of CED-9(n1950) to interact with EGL-1. Instead, it could be hypothesized that upon EGL-1 binding, CED-9 adopts a pro-apoptotic function, which is required for mitochondrial fragmentation in apoptotic cells. For example, could CED-9 be involved in the recruitment of DRP-1 to the mitochondria allowing for mitochondria to fragment, which is required for the progression of death? It is also possible that CED-9 might have a more general role in mitochondrial fission. To determine the role of CED-9 in the regulation of mitochondrial fragmentation during cell death I determined whether egl-1- or drp-1-induced mitochondrial fragmentation is blocked in a *ced-9(lf)* background (see section 2.5).

How is it that *egl-1*, which seems to be expressed predominantly in cells destined to die, is required for the ectopic death of cells destined to survive?

egl-1 has been thought to exclusively function in cells destined to die. Consequently, it was presumed not to be expressed to any appreciable levels in cells destined to survive. This hypothesis is based on the following findings: 1) egl-*1(lf)* mutations display no overt phenotype besides a complete block in programmed cell death (Conradt and Horvitz, 1998). 2) Through promoter analysis, *egl-1* appears to be predominantly expressed in cells destined to die (Conradt and Horvitz, 1999; Thellmann et al., 2003).

It is conceivable that *egl-1* might be expressed at very low levels in cells destined to survive and thus all cells within the embryos. This low expression might not have been detected by GFP based promoter analysis. A low level of expression could be sufficient to mediate the requirement for egl-1 in *drp-1*-induced cell death. This hypothesis is supported by the fact that *egl-1(lf)* animals have a defect in cell death in cells destined to survive (Barbara Conradt, unpublished data). In light of these results it can be concluded that it is likely that e*gl-1* is expressed at a low levels in all cells in the embryo. In mammalian cells, BH3-only proteins can be sequestered, sometime inactively attached to the cytoskeleton, in the absence of cell death stimuli (Huang and Strasser, 2000). It is conceivable that a sequestered pool of EGL-1 may also exist in all cells in the embryo to maybe mediate some currently uncharacterized cell death event.

2.5 Mitochondrial morphology changes upon induction of *egl-1* and *drp-1* in *ced-9(lf)*

CED-9 had been previously shown to have a killing function (Hengartner and Horvitz, 1994b). This was concluded by the observation that *ced-9*(*n2812*lf) enhances the block in cell death caused by weak *ced-3*(lf) mutations (Hengartner and Horvitz, 1994b). Could it be that *ced-9*'s killing function involves mitochondrial fragmentation, which is inactivated by the *ced-9*(*n1950*gf) mutation? Furthermore, the blockage of mitochondrial fragmentation in *ced-9*(*n1950*gf) animals could also be a consequence of CED-4 irreversibly binding to mitochondria through its interaction with CED-9(n1950) (Chen et al., 2000). CED-4 and not CED-9 would then possibly be involved in mitochondrial fragmentation in apoptotic cells. To help elucidate the killing function of *ced-9*, I determined whether *egl-1*- or *drp-1*-induced mitochondrial fragmentation occurs in a *ced-9*(*n2812*lf) background.

Because of the loss of CED-9's anti-apoptotic function, *ced-9*(*n2812*lf) animals are not viable as a result of ectopic programmed cell death (Hengartner and Horvitz, 1994a). In order to examine *egl-1*- or *drp-1*-induced mitochondrial fragmentation in the absence of the CED-9 protein, the experiments were performed in the background of the mutation *ced-3*(*n717*). This was possible for two reasons: 1) *ced-3* acts downstream of *ced-9*, so the loss of *ced-3* function blocks *ced-9*(*n2812*lf)-induced ectopic cell death and thus *ced-9*(*n2812*lf)-induced lethality. 2) It was previously determined that *ced-3*(*n717*) does not affect the ability of EGL-1 or DRP-1 to induce mitochondrial fragmentation (Fig.19 and 22), so if any effects are observed, it is as a consequence of the loss of CED-9 protein.

Before I could determine whether mitochondria fragment after expression of *egl-1* and *drp-1*, it was important to ensure that the double mutants did not display an abnormal mitochondrial morphology. Based on rhodamine staining, *ced-9*(*n2812*lf); *ced-3*(*n717*) embryos appeared to have a normal mitochondrial morphology and distribution (Fig.23). This suggests that after loss of CED-9 protein, mitochondria are respiring, maintaining a potential and are not significantly abnormal in shape or size.

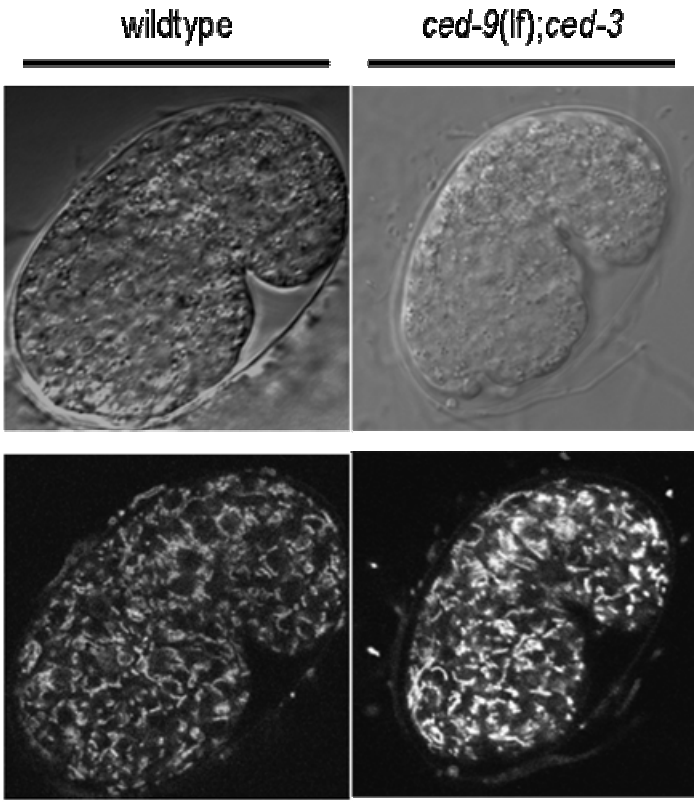

Figure 23. *ced-9(n2812*lf) does not affect mitochondrial morphology in general.
Representative DIC (top) and rhodamine (bottom) images of wild-type, and *ced-9*(*n2812*lf); *ced-3*(*n717*) animals, at the comma to 1 ½-fold stage of embryonic development. Mitochondria were stained with rhodamine B hexyl ester as described in Methods. Images of rhodamine-stained embryos represent single confocal planes.

Mitochondria were then imaged in embryos of *ced-9*(*n2812*lf); *ced-3*(*n717*) and *ced-3*(*n717*) animals 2 hours after the initial heat shock, which induced the transgenes (P_{hs}*drp-1*(wt) and P_{hs}*mitogfp* or P_{hs}*egl-1* and P_{hs}*mitogfp*). Two hours of heat shock ensured that the transgene expression had enough time to take effect, following previously determined time kinetics for both *egl-1*- and *drp-1*-induced mitochondrial fragmentation (see sections 2.2 and 2.4, respectively). It was found that, like *ced-9*(*n1950*gf), *ced-9*(*n2812*lf) blocked the ability of both EGL-1 (Fig.24, 2nd panel from left) or DRP-1 (Fig.24, 4th panel from left) to induce mitochondrial fragmentation. In contrast, as previously determined, control *ced-3(717)* embryos appeared highly fragmented after induction of either cell death stimuli (Fig.24, 1st and 3rd panel from the left). This result demonstrated that a functional CED-9 protein is required for mitochondrial fragmentation after death induction. These findings support the model that *ced-9*(*n1950*gf)'s ability to block *egl-1*- and *drp-1*-induced mitochondrial fragmentation is a result neither of its inability to interact with EGL-1 nor its inability to release CED-4.

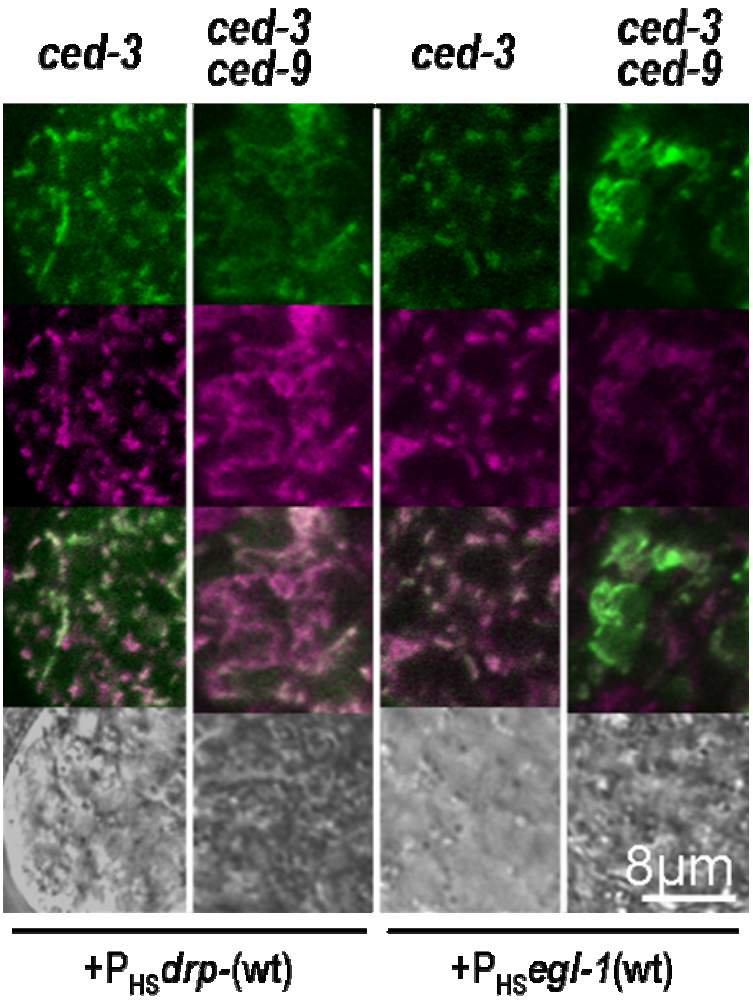

Figure 24. *ced-9(n2812*lf) blocks the ability of EGL-1 and DRP-1 to induce mitochondrial fragmentation.
Representative confocal images are shown of mitoGFP, rhodamine, mitoGFP/rhodamine overlay and DIC (from top to bottom) of *ced-3(n717*) animals (first and third columns) or *ced-9(n2812*lf); *ced-3(n717)* animals (second and fourth columns) carrying a P_{hs}*drp-1*(wt) and P_{hs}*mitogfp* transgene (first and second columns) or P_{hs}*egl-1* and P_{hs}*mitogfp* transgenes (third and fourth columns). Transgenes were induced as described in Methods, and embryos were imaged at the comma to 1½-fold stage of embryonic development. Images represent single confocal image planes. Scale bar, 8 µm.

From these observations it was concluded that *ced-9*'s killing function in cells destined to die is synonymous to *ced-9*'s role in mitochondrial fragmentation in apoptotic cells. However, CED-9's mechanistic role in mitochondrial division remains an enigma. Of interest, in mammalian cells it was observed that endogenous Drp1 co-localizes with Bax, a pro-apoptotic Bcl-2 family member, on the mitochondria after induction of cell death (Karbowski et al., 2002). Bax might therefore be directly involved in the activation of mitochondrial fragmentation during apoptosis. Since CED-9 contains the BH regions of Bax and appears to be structurally similar, CED-9 might be functionally homologous to Bax with respect to promoting cell death. It might be possible that binding of EGL-1 to the mitochondrial CED-9 and/or release of CED-4 from the mitochondrial CED-9 leads to CED-9 adopting a Bax-like killing function.

2.6 Does *ced-9* affect the localization EGL-1 and/or DRP-1?

EGL-1 is required to induce mitochondrial fragmentation in apoptotic cells and CED-9's killing function is required for this fragmentation event. How endogenous DRP-1 is involved in this step is unclear but it appears that CED-9's killing function is mediated through DRP-1-induced mitochondrial fragmentation. At the time of death, are both EGL-1 and DRP-1 recruited to the mitochondria through CED-9? Or does EGL-1 get initially recruited to the mitochondria through CED-9, concomitantly unmasking a novel

function of CED-9, which then results in the recruitment of DRP-1 to the mitochondria? Is CED-9 involved in the recruitment of either endogenous EGL-1 and/or DRP-1? To answer these important questions requires determining whether DRP-1 physically interacts with either or both EGL-1 and CED-9, whether DRP-1 co-localizes with either EGL-1 or CED-9, and whether the sub-cellular localization of DRP-1 and EGL-1 is affected by CED-9. To resolve these issues requires a detailed biochemical analysis of the various interactions *in vitro* and also *in vivo*. As well structural analysis will be required to unravel the dynamics of how CED-9 structurally adopts a killing function upon EGL-1 binding. To address the latter issue, transgenic lines overexpressing the following fusion proteins were used: DRP-1-GFP under the control of its endogenous promoter (P_{drp-1}*drp-1-gfp)* and GFP-1-EGL-1 under the heat shock promoter (*P_{hs}gfp-egl-1*). It had previously been determined that the GFP-EGL-1 fusion protein is functional *in vivo* (Barbara Conradt, unpublished data). However, it remains to be determined whether the DRP-1-GFP fusion protein is functional *in vivo*.

2.6.1 Subcellular localization of DRP-1

Since the GTPase domain of DRP-1 is at the N terminus of the protein, and N-terminal fusion proteins of DRP-1 behave like dominantly interfering proteins (van der Bliek, unpublished observation), I attempted to address DRP-1 subcellular localization using a C-terminal GFP fusion protein. The transgene P_{drp-1}*drp-1-gfp* was expressed in wild-type, *ced-9(1950*gf*)* and *ced-9(2812*lf*)* animals in order to determine if CED-9 affected DRP-1 localization. Unfortunately, this approach was flawed, because *drp-1-gfp* expression, in

some cases, was acting like the dominant negative drp-1(K40A) gene (Fig. 25b). In wild-type embryos, DRP-1-GFP appeared to distribute along the mitochondria in punctate patches "hotspots", with very little GFP signal detectable in the cytosol (Fig. 25a). Recent work in yeast supports this finding. It appears that most of the Dnm1p-GFP pool is associated with the mitochondria (Astrid Schauβ and Stefan Jakobs, unpublished results). Two lines of evidence suggest that this fusion protein was exerting a dominant negative effect. In some cases, it appeared that mitochondria were highly aggregated and not evenly distributed, and that GFP was highly enriched within the aggregated mitochondria (Fig 25b). Furthermore, like the inhibitory effect of *drp-1*(K40A) expression on cell death, *drp-1-gfp* expression led to the inappropriate survival of 1 to 2 extra cells in the anterior pharynx of about 10% of transgenic animals ("Extra cells" counted by Barbara Conradt). For this reason, it was not possible to further analyze the differences between localization of the fusion protein in the various backgrounds. However, preliminary results suggest that like in wild-type animals, DRP-1-GFP localized in punctate patches along the mitochondria in *ced-9(1950*gf*)* animals (Fig. 25a). Therefore, it is likely that any affect of *ced-9(1950*gf*)* might have on the subcellular distribution of DRP-1 are more subtle than a complete loss of mitochondrial targeting.

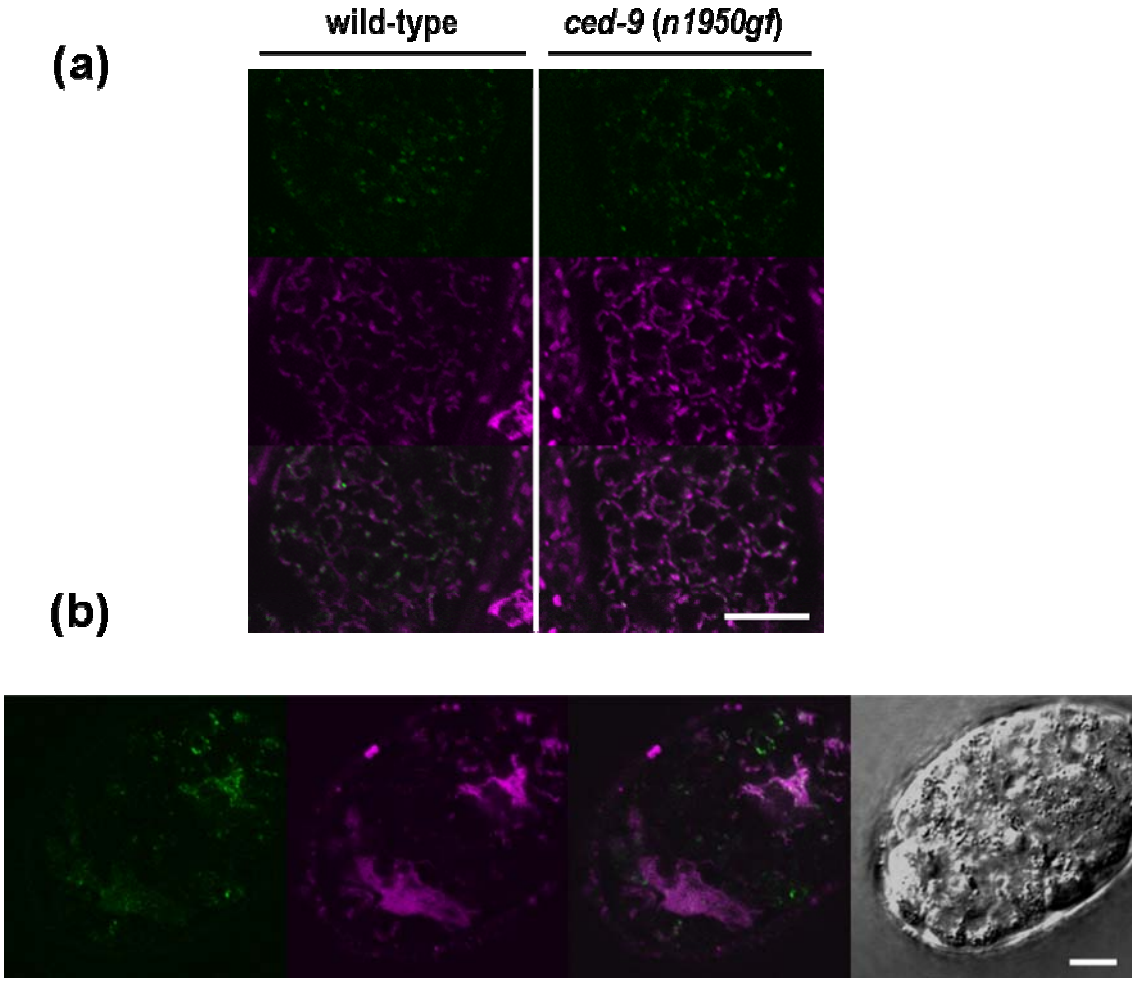

Figure 25. DRP-1-GFP localizes to mitochondria, and in some cases exerts a dominant negative effect.
(a) Representative confocal images are shown of mitoGFP, rhodamine, mitoGFP/rhodamine overlay (from top to bottom) of wild-type (left panel), and *ced-9(n1950*gf) (right panel) animals carrying a P_{drp-1}-*drp-1-gfp* transgene. Embryos were imaged at the comma to 1½-fold stage of embryonic development. Images represent single confocal image planes. Scale bar, 8 µm. (b) Representative confocal images of mitoGFP, rhodamine, mitoGFP/rhodamine overlay and DIC (from left to right) of wild-type embryo, in which the P_{drp-1}-*drp-1-gfp* transgene exerts a dominant negative effect. Scale bar, 8 µm.

2.6.2 Subcellular localization of EGL-1

To address EGL-1 subcellular localization, a C terminal GFP fusion protein was expressed under the control of the heat shock promoter (Barbara Conradt, unpublished observation). Most likely because of low levels of endogenous protein, so far only overexpressed but not endogenous EGL-1 protein is detectable using an EGL-1 specific antibody (Barbara Conradt, unpublished observation). The transgene P_{hs}*egl-1-gfp* was expressed in an *egl-1(n3330)* background and stable integrated transgenes were established. One of these stable transgenes (referred to as "*bcIs16*") was then crossed into the following genetic backgrounds: *ced-9(n1950*gf); *egl-1(n3330)*, *ced-9(n2812*lf); *egl-1(n3330)*, *ced-3(n717); egl-1(n3330),* and *ced-4(n1162)*; *egl-1(n3330)* (Barbara Conradt, unpublished observation). The EGL-1-GFP fusion protein was at least partially functional since it retains its killing function. Two hours after heat shock, there was an increased number of cell corpses in transgenic embryos (10-15 per embryo, n=13) compared to wild-type embryos (1-2 per embryo, n=17). However, the increase cell death is far less than what is observed with the wild-type EGL-1 protein. Ectopic corpses (>30 per embryo) are usually present within 2 hours of a 45 minute heat shock of the wild-type *egl-1* protein.

EGL-1-GFP appeared to localize to the mitochondria in *egl-1(n3330)* animals. Upon expression, EGL-1-GFP localized to mitochondria and, similar to wild-type EGL-1,

induced the organelles to fragment (Fig.26, left panel). Furthermore, in both *ced-4(1162)* and *ced-3(717)* animals (Fig.26, 4th and 5th panel from the left), upon expression of the fusion protein, GFP localized to the mitochondria. This observation is consistent with the assumption that *egl-1*-induced mitochondrial fragmentation requires translocation of the protein to the mitochondria, since it was previously observed that *egl-1*-induced mitochondrial fragmentation was independent of *ced-3* and *ced-4* (Fig.16). Furthermore, mitochondria appear to fragment after the induction of GFP-EGL-1 expression in *ced-4(1162)* and *ced-3(717)* embryos (data not shown). Interestingly, in both *ced-9(1950*gf*)* and *ced-9(n2812*lf) mutant embryos overexpressed EGL-1-GFP showed a highly variable expression pattern. In both backgrounds the same general trend was observed, lower level of *gfp* expression, increased GFP signal in the cytosol but "hot spots" or patches of fluorescent protein were seen in close proximity to the mitochondria (Fig. 26, 6th and 7th panel from the left). Since the expression pattern was both lower and variable, it is hard to draw any conclusions from these observations. However, the high level of cytoplasmic EGL-1-GFP could indicate that the CED-9 protein is required for a stable association of EGL-1 with the mitochondria and thus the low level of overall expression could be as a result of degradation of the fusion protein in the cytosol.

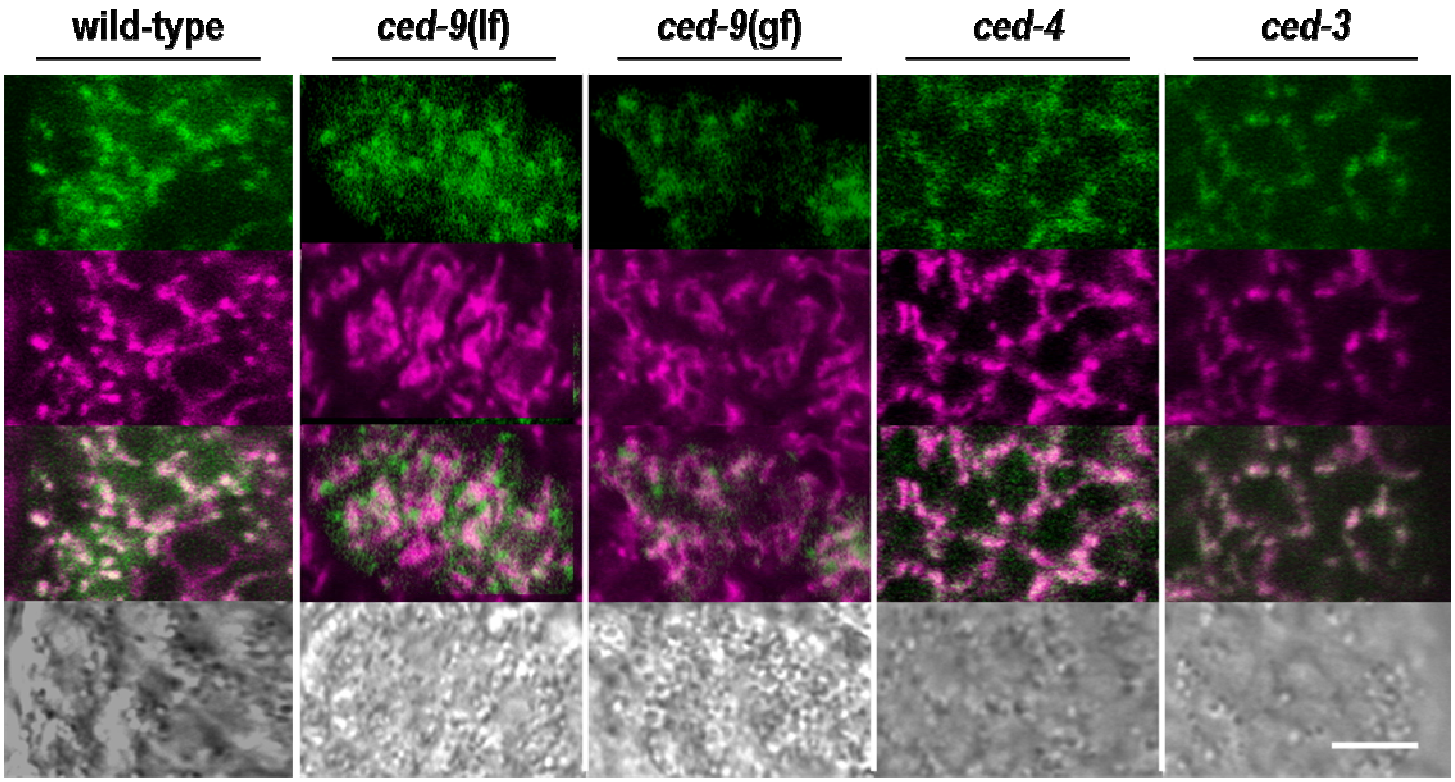

Figure 26. GFP-EGL-1 appears to, at least partially, localize to the mitochondria in all background examined.
Representative confocal images are shown of mitoGFP, rhodamine, mitoGFP/rhodamine overlay and DIC (from top to bottom) of *egl-1(n3330)* animals carrying the stable transgene P_{hs}*egl-1-gfp* transgene (*bcIs16*) alone (control, first column), as well as *ced-9(n2812*lf); *egl-1(n3330)*, *ced-9(n1950*gf); *egl-1(n3330)*, *ced-3(n717); egl-1(n3330)* or *ced-4(n1162); egl-1(n3330)* animals carrying *bcIs16* (columns, as indicated). After induction of the transgenes, embryos were imaged at the comma to 1½-fold stage of embryonic development. Images represent single confocal image planes. Scale bar, 8 μm.

3 Future perspectives

In summary of the results presented in this thesis, an important role can be assigned to mitochondria in programmed cell death in *C. elegans*. These results provide the first clear evidence that mitochondrial fragmentation is involved in the early stage of programmed cell death in *C. elegans*. What could the need for mitochondrial fragmentation be in the apoptotic process? It is likely that a pro-apoptotic factor or factors capable of enhancing the activity of the central cell death machinery (*egl-1*, *ced-4*, *ced-3*) is released from *C. elegans* mitochondria early during the apoptotic process. Studies on the *C. elegans* homologues of EndoG and AIF, namely CPS-6 and WAH-1, respectively, have indicated a possible role and release of mitochondrial proteins late in the apoptotic process in *C. elegans* after CED-3 activation (Parrish et al., 2001; Wang et al., 2002). Therefore, I hypothesized that a mitochondrial fragmentation step may facilitate the release of these apoptogenic factors and, others, such as Cytochrome *c* from the inter membrane space.

It had previously been observed that mitochondria fragment into small units early during apoptosis in a variety of mammalian systems through the Drp1-mediated division pathway (Frank et al., 2001; Breckenridge et al., 2003). The work presented here demonstrates that mitochondria fragment during physiological cell death in *C. elegans* via this same cellular machinery. Interestingly, Drp-1-mediated mitochondrial fragmentation during cell death was recently documented in yeast cell death (Frank et al.,

2001; Fannjiang et al., 2004; Pozniakovsky et al., 2005). The common theme among these evolutionary distinct eukaryotes is that inhibition of the fragmentation machinery can impede cell death. Therefore, mitochondrial fragmentation may reflect the underlying mechanism of caspase activation that occurs in all eukaryotes, unifying the currently fragmented hypotheses in the field.

From these findings, the programmed cell death machinery in *C .elegans* needs to be re-evaluated, as the current model comprising the four core components (*egl-1, ced-9, ced-4,* and *ced-3*) is incomplete. This study demonstrates that upon *egl-1* transcription, mitochondria fragment through a previously uncharacterized component, *drp-1,* which in turn promotes killing by enhancing the activity of *ced-4* and/or *ced-3*'s killing functions (see Fig. 27a and 28). In cells destined to survive, *egl-1* is not transcribed, *drp-1* is not activated on the mitochondria, mitochondria do not fragment and *ced-4* is not released (Fig. 27a,).

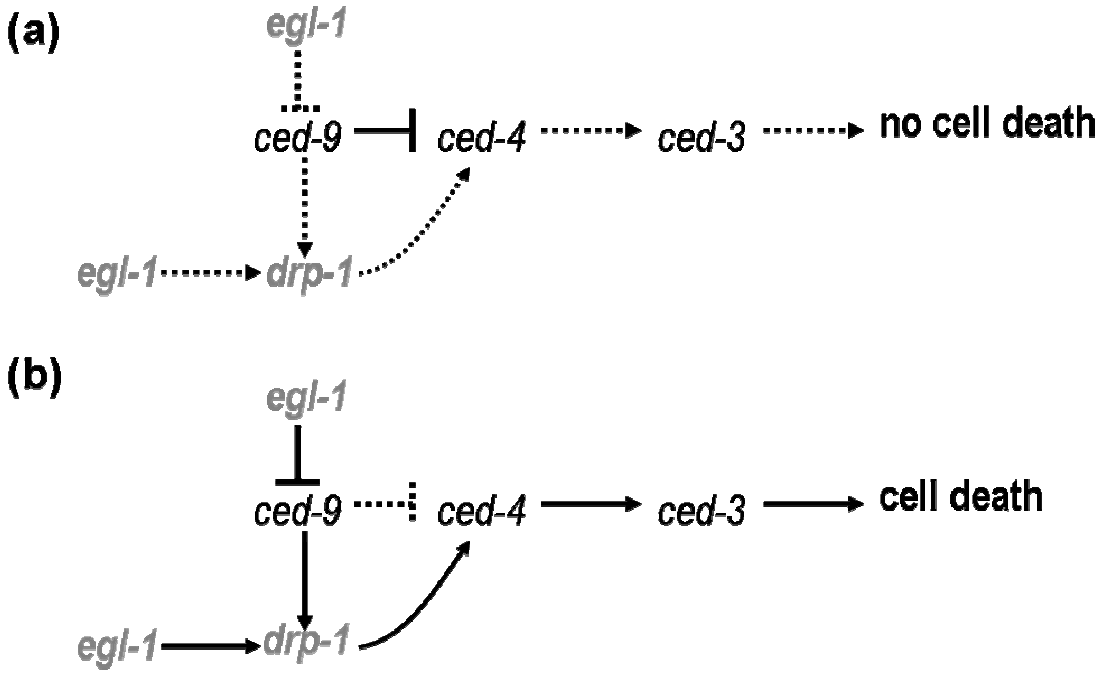

Figure 27. A genetic pathway for the activation of programmed cell death during *C. elegans* development.
(a) In healthy cells, the anti-apoptotic function of *ced-9* prevents the activation of programmed cell death by negatively regulating the pro-apoptotic gene *ced-4*. (b) In cells destined to die, *egl-1* blocks the anti-apoptotic function of *ced-9*, thereby activating *ced-4* and *ced-3*, which can result in programmed cell death. *egl-1* also activates the pro-apoptotic killing function of *ced-9*, thereby inducing *drp-1* activity, which contributes to programmed cell death by enhancing *ced-4* and *ced-3* activity. (Jagasia et al., 2005). Solid arrows and T-bars indicate activation and suppression respectively, and dotted arrows and T-bars indicate suppression and activation respectively that did not occur because the cell either lived or died.

This study implicates a new player in *C. elegans* physiological cell death, *drp-1,* which is likely recruited and/or activated on the mitochondria where it mediates mitochondrial fragmentation (Fig.28). Furthermore, through genetic analyses, this study identified a novel functions for both CED-9 and EGL-1 during apoptosis. EGL-1 binding to CED-9 likely unmasks a killing function for CED-9; the killing mechanism somehow results in DRP-1 activation and subsequent mitochondrial fragmentation (Fig 28). Loss of EGL-1 function does not inhibit the mitochondrial fragmentation whereas the loss of CED-9 function does, implying that CED-9 can function independently of EGL-1 in the fragmentation step once the process has been initiated by EGL-1. However, fragmentation alone can not drive the apoptotic process, since DRP-1-induced cell death but not mitochondrial fragmentation is egl-1 dependent. Thus, binding and formation of an EGL-1/CED-9 complex leads to more than just fragmentation through DRP-1activation. It is known that EGL-1 is required so that some CED-4 can be released from the mitochondria and then subsequently activate CED-3 (Chen et al., 2000). Could EGL-1 mediate another function on the mitochondria during killing? It is conceivable that EGL-1 may induce CED-9 to form a pore in the outer membrane, which would allow for the release of apoptogenic substances from the inter membrane space. Increasing the permeability of the outer membrane may compromise the function of mitochondria, requiring the generation of independent mitochondria through fragmentation prior to this step.

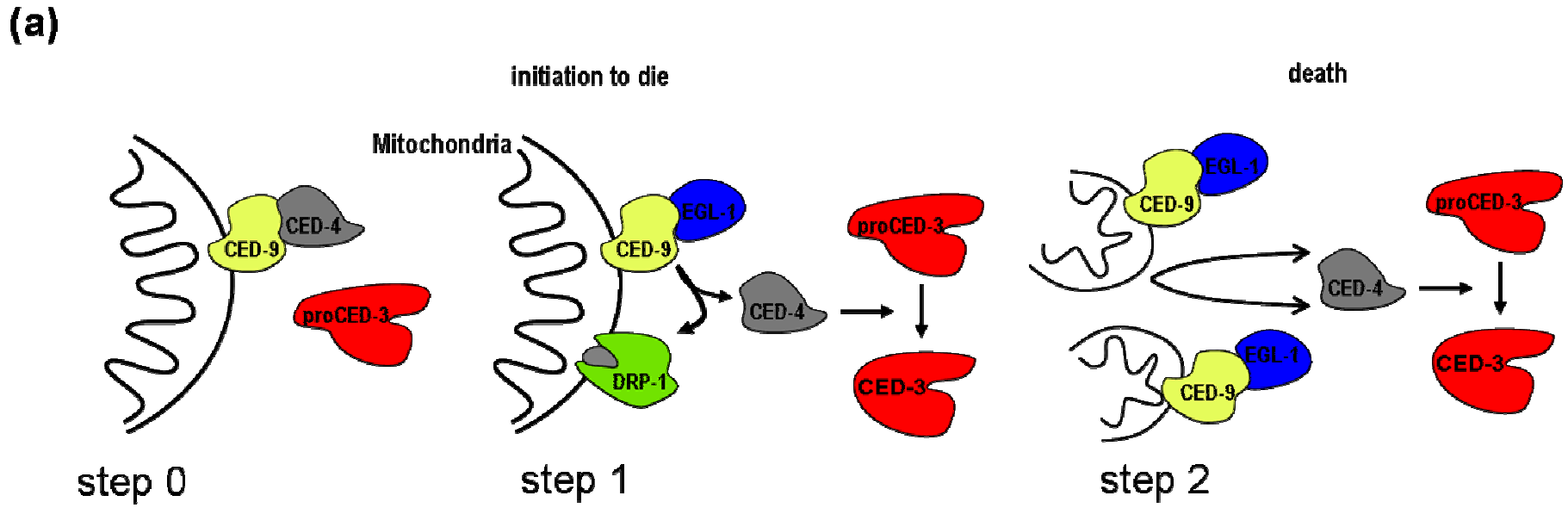

Figure 28. A simplified model for the molecular interactions occurring during the activation of programmed cell death, viewed in a stepwise process (step 0-2).
Before initiation of cell death (living cell, step 0), CED-9 blocks CED-4's ability to activate proCED-3. After EGL-1is transcribed (initiation to die, step 1), EGL-1binds to CED-9 in cells causing the release of CED-4 from the mitochondria allowing CED-4 to activate proCED-3, which can result in programmed cell death (step 1). EGL-1 binding to CED-9 also activates CED-9's killing function, resulting in DRP-1 activation and DRP-1-mediated mitochondrial fragmentation (step 1). Mitochondrial fragmentation contributes to programmed cell death by potentiating CED-4 activity and proCED-3 activation (death, step 2). Alternatively, the pro-apoptotic activity of DRP-1 could be a function of DRP-1 that is independent of its function in mitochondrial fragmentation. (Metzstein et al., 1998; Jagasia et al., 2005)

This work provides a better understanding of how the *C. elegans* core cell death machinery operates. However, the full story most likely is far from being complete. An answer to the fundamental question why the CED-9/CED-4 complex is located on the outer mitochondrial membrane can finally be addressed. The CED-9/CED-4 complex is located on the outer mitochondrial membrane because mitochondria are actively involved in programmed cell death. However this newly revised molecular model creates many questions: How might mitochondrial fragmentation contribute to killing? Could mitochondrial fusion as well regulate cell death? How is mitochondrial fragmentation regulated or activated during cell death**?**

3.1 How is mitochondrial fragmentation regulated or activated during cell death?

As described above (Fig.28), during physiological cell death, EGL-1 initiates a cascade of events that leads to DRP-1-mediated mitochondrial fragmentation in a CED-9-dependent manner. How do EGL-1 and CED-9 function to catalyze this reaction? What is known is that concomitantly with the loss of a CED-4/CED-9 complex an EGL-1-CED-9 complex is formed in the dying cell. It is therefore possible that it is this newly formed complex rather than EGL-1 alone that activates mitochondrial fragmentation. In support of this possibility, it was shown that CED-9 could directly regulate DRP-1 function at the mitochondria. To better understand these questions will require biochemical approaches to determine whether EGl-1/CED-9/ DRP-1 co-exist in a complex in dying cells, or if

either CED-9 or EGL-1 physically interacts with DRP-1. To further address how the EGL-1/CED-9 complex causes CED-9 to adopt a killing function will likely require *in vitro* reconstitution of at least part of the cell death process using purified *C. elegans* mitochondria. This would also make it possible to determine whether CED-9 has channel like activity upon EGL-1 binding.

To complicate matters, the *ced-4* gene encodes two alternatively spliced mRNAs, called *ced-4L* and *ced-4S*, which have been shown to have opposing functions (Shaham and Horvitz, 1996b). ced-4L has been shown to confer a protective function during cell death. It is possible that the loss of the CED-4/CED-9 complex causes mitochondrial fragmentation and that the *ced-4* cell-death protective function antagonizes mitochondrial fragmentation. It will be interesting to determine whether overexpressing either isoform can induce or block mitochondrial fragmentation or, conversely, mitochondrial fusion. Furthermore, the *ced-4* mutation *n2273*, which has been proposed to predominantly result in the loss of the CED-4 protective function, is synthetically lethal with the weak ced-9 loss-of-function mutation n1653 (Shaham and Horvitz, 1996b). Could CED-4's pro-survival function be to inhibit CED-9/DRP-1-mediated mitochondrial fragmentation? Alternatively, CED-4's pro-survival function maybe to increase mitochondrial fusion, thereby offsetting DRP-1-mediated mitochondrial fragmentation.

Since DRP-1 requires other components to induce mitochondrial fission (see section 1.6), it is likely that these components are also involved in DRP-1 actions during physiological cell death. Studies in yeast indicate that at least 3 proteins, Dnm1p, Fis1p and Mdv1p,

are involved in outer-membrane fission. Furthermore, an additional set of proteins is required to coordinate fusion and fission events involving the two membranes. In *C. elegans* two homologues of yeast Fis1p were identified, which were named *fis-1* and *fis-2*, and a putative homologue for Mdv1p may exist (Dr. van der Bliek, personal communication). In yeast, Mdv1p promotes cell death (Fannjiang et al., 2004). It could be that the *C. elegans* homologue serves the same function. 48 hours after the overexpression of hFis1, the human orthologue of yeast *FIS1*, mitochondrial fragmentation, Cytochrome *c* release and death was observed (James et al., 2003). However, caution should be taken in interpreting these results, since the effects observed may not have been apoptosis and merely mitochondrial stress. Opposite findings come from yeast, where overexpressing of *FIS1* impedes cell death (Fannjiang et al., 2004). Could *C. elegans fis-1* and *fis-2* be involved in programmed cell death? It will be interesting to determine whether overexpressing the *fis* genes in *C. elegans* will induce both mitochondrial fragmentation and death. Conversely, will animals lacking a functional *fis-1* and/or *fis-2* gene have defects in cell death? It is possible that mutations in these two genes were not identified through genetic cell death screens because they have partially redundant functions. Even more puzzling is the question why *C. elegans* requires two homologues, whereas in yeast and mammals a single protein is sufficient.

3.2 Could mitochondrial fusion as well regulate cell death?

Strong evidence from the mammalian field is emerging, indicating that a block in fusion can as well trigger the induction of apoptosis (see section 1.8). In this study it was

determined that simply overexpressing *drp-1* and inducing mitochondrial fragmentation caused cell death. This fragmentation step triggered but did not cause programmed cell death without the existence of functional cell death proteins. Is it possible that fragmentation caused by a block in fusion leads to the same outcome? Is it possible to induce ectopic cell death by simply blocking mitochondrial fusion? If that was the case, the only function of *drp*-1 in programmed cell death would be to induce mitochondrial fragmentation. To determine the effects of inhibiting mitochondrial fusion on *C. elegans* cell death, one could performed RNAi experiments or analyze mutants defective in mitochondrial fusion.

Studies in yeast have shown that two large GTPases, Fzo1p and Mgm1p, regulate mitochondrial fusion (see Section 1.7). The *C. elegans* genome contains genes encoding homologues of both proteins and deletion alleles, *tm1133* and *tm1107*, exist, respectively. Both alleles appear to be functional nulls. Interestingly, *mgm-1*(tm1107) but not *fzo-1*(tm1133) causes lethality. Is it possible that *mgm1* embryos die from excessive programmed cell death? If this was the case, it would be consistent with the finding that mutations in *OPA1*, the human MGM-1 homologue, cause excessive apoptosis in the retina of patients (Alexander et al., 2000; Delettre et al., 2002). Furthermore, the downregulating of endogenous OPA1 leads to apoptosis in mammalian cells (Olichon et al., 2003). So it will be interesting to determine whether the embryonic lethality caused by *tm1107* can be suppressed by mutations for example of ced-3 or ced-4.

Another interesting observation from the mammalian field is that both fission and fusion components colocalize with the pro-apoptotic molecule Bax on mitochondria (Karbowski

et al., 2002). Could the fusion machinery be involved in inhibiting Bax-induced apoptosis, maybe by counterbalancing Bax-induced fission? If fusion proteins could directly inhibit Bax-mediated apoptosis, it would present another level of apoptosis regulation. To study whether mitochondrial fusion can negatively regulate physiological cell death in *C. elegans* can be easily investigated. This can be achieved by overexpressing mitochondrial fusion proteins in cells destined to die, similar to the overexpressing of *drp-1*(K40A), and by determining if this perturbation can inhibit physiological cell death.

3.3 How might mitochondrial fragmentation contribute to killing?

This study showed that *drp-1*-induced cell death is dependent on *egl-1*, *ced-4*, and *ced-3*, and that *drp-1*-induced-mitochondrial fragmentation is dependant on *ced-9*. It can therefore be hypothesized that mitochondrial fragmentation affects cell death by acting through the genes *egl-1*, *ced-4*, and *ced-3*. The mitochondrial fragmentation pathway might therefore enhance the central cell death pathway or act like an amplification loop of the central pathway (see Figure 27 and 28). To determine how mitochondrial fragmentation contributes to programmed cell death two approaches can be taken: 1) Determine how fragmentation affects the function of these genes. 2) Determine what the role of fragmentation is during cell death.

In order to examine what *drp-1*-induced-mitochondrial fragmentation does to the cell death genes/protein, the expression levels, sub cellular localizations, posttranslational modifications before and after *drp-1* induction could be compared. Important questions to answer include: Does CED-4 translocation from mitochondria require mitochondria to fragment a prior? Does CED-9 get processed, maybe through cleavage, and if so before or after fragmentation? Does CED-3 processing and activity require mitochondrial fragmentation?

As mentioned above, it is hypothesized that mitochondrial fragmentation somehow enables the release of pro-apoptotic factors from the inter-membrane space. In order to identify these putative pro-apoptotic factors, one can take advantage of the *in vivo* system established and presented here. The ideal experiment is as follows: create fusion proteins of various inter membrane space proteins with fluorescent protein variants, proteins such as WAH-1, Cytochrome *c* and non cell death associated proteins; express these proteins in *C. elegans* embryos before cell death; watch whether these fusion proteins translocate from the mitochondria to the cytoplasm during physiological-, *egl-1*- and *drp-1*-induced cell death. If release is seen, determine if release is restricted to cell death associated proteins. Determine if release can be blocked by blocking mitochondrial fission by overexpressing *drp-1*(K40A). This would surely provide clear evidence for whether this hypothesis holds water.

4 Final Remarks

During the time this work was initiated, I have had the pleasure of seeing an expansion of the field of mitochondrial dynamics and its possible role in cell death. That mitochondria fragment during cell death is an emerging concept and the studies presented here provide evidence that mitochondria are equally dynamic in apoptotic cells in *C. elegans*. Through the use of the *in vivo* system that I have established, additional, new insight can be gained in the future. The most important advantage of this system is that physiological cell death can be studied *in vivo* in real time. This fact provides a significant advantage to the study of how mitochondria regulate cell death.

5 Materials and methods

5.1 Growth media and *C. elegans* strains

Worms were grown on nematode growth media (NGM) at 20°C or 15°C as described previously (Brenner, 1974). To make 1 l of NGM medium 3 g NaCl, 17 g bacto-agar, 2.5 g bacto-peptone and 975 ml ddH_2O were autoclaved, and then 1 ml cholesterol (5 mg/ml in 95% EtOH), 1 ml of sterile 1 M $CaCl_2$, 1 ml of sterile 1 M $MgSO_4$, 25 ml of sterile 1 M KH_2PO_4 pH 6.0 was added. After NGM plates solidified, *Escherichia coli* strain OP50, was dropped onto the plate to serve as worm food. Seeded plates were incubated for a day at room temperature, and then could be used for several weeks.

C. elegans strains were cultured as described by Brenner, 1974. The wild-type strain was N2. The following alleles were described previously: *ced-3(n717)* has been described by Yuan et al., 1993, *ced-4(n1162)* has been described by Yuan and Horvitz, 1992, *ced-9(n1950)* has been described by Hengartner et al., 1992, *ced-9(n2812)* has been described by Hengartner et al., 1992, *egl-1(n1084n3082)* has been described by Conradt and Horvitz, 1998, *ced-1(e1735)* has been described by Zhou et al., 2001, *ced-3(n2427)* has been described by Shaham et al., 1999, and *rrf-3(pk1426)* is described by Simmer et al., 2002. The following alleles were not described previously:
Newly created transgenic lines (see Section 5.3), newly created *drp-1(bc259)* (see section 6.4), newly created egl-1(n3330) (Isolated by Barbara Conradt) and newly created

stable integrations, *bcIs49* (see Section 6.3), and *bcIs16* (Done by Barbara Conradt), containing plasmids P_{hs}*egl-1-gfp* and P*lin-15+*.

5.2 Molecular biology

mitoGFP was amplified by polymerase chain reaction from the plasmid p_{myo-3} *mitoGFP* which was previously described (Labrousse et al., 1999), cloned blunt into pBluescript using the *Eco*RV restriction site. *mitoGFP* was then subcloned to replace *gfp* in pBC99, a plasmid previously described (Conradt and Horvitz, 1999) using *Xma*I and *Spe*I for to create P_{egl-1}*mitogfp*. In addition pBluescript-*mitoGFP* was subcloned into pPD49.78 and pPD49.83 (*C. elegans* vectors for heat-inducible expression) using *Bam*HI and *Spe*I to create P_{hs}*mitogfp*, respectively. The pPD49.78-based and pPD49.83-based plasmids were injected together and are referred to as P_{hs}*drp-1*(wt) and P_{hs}*drp-1*(K40A). These plasmids were created by subcloning from plasmids P_{myo-3}*drp-1* and P_{myo-3}*drp-1(K40A)*, previously described (Labrousse et al., 1999), into pBluescript using *Bam*HI and *Spe*I. The pBluescript plasmids were then subcloned into pPD49.78 and pPD49.83 using *Bam*HI and *Spe*I to create P_{hs}*drp-1* andP_{hs}*drp-1*(*K40A*). In addition the pBluescript plasmids were then subcloned into pBC99 using *Xma*I and *Spe*I to create P_{egl-1}*drp-1*(*K40A*). P_{hs}*egl-1* (pBC27 and pBC28) were described previously (Conradt and Horvitz, 1998).

5.3 Transgenic animals

Germline transformation experiments were performed using standard methods (Mello and Fire, 1995). To create transgenic line carrying specific combinations of plasmids, two different markers were used to assist in identifying transformed animals. 1) Plasmids of interest were injected at 5ng/µl into *unc-76*(*e911*) mutants using the *unc-76*(+) rescuing plasmid, p76-16B, as a marker as previously described (Bloom and Horvitz, 1997). Therefore, non-transgenic animals can be identified by their uncoordinated movement (Unc) and transgenic, rescued animals will appear wild-type or "non-Unc". 2) Plasmids of interest were injected into the wild-type by using the plasmid pRF4 (Mello and Fire, 1995). pRF4 contains a dominant mutation in the rol-6 gene interfering with the collagen structure of the cuticle in *C. elegans*, causing worms to roll as opposed to the wild-type sinusoidal movement. Therefore, non-transgenic animals will appear wild-type and transgenic animals will confer a Rol (for roller) phenotype. After injection, transgenic non-Unc or Rol F1 progeny were picked and used to establish stable transgenic lines.

Since the expression of the P_{egl-1}*mitogfp* transgene after germline transformation was low and variable, a stable line was generated to enhance the expression. In order to integrate P_{egl-1}*mitogfp* transgene and *unc-76*(+), animals carrying the P_{egl-1}*mitogfp* transgene, non-Unc, were mutagenized with ethylmethanesulphonate. A stable integrant, *bcIs49*, was identified by screening for F2 animals that transmitted the transgene to 100% of their progeny. To analyze the expression of *bcIs49* in mutant backgrounds, *bcIs49* was crossed into *ced-3(n717), ced-3(n2427), ced-4(n1162), ced-9(n1950*gf*), egl-1(n1084n3082).*

5.4 Construction of *drp-1(bc259)* strains

To isolate a deletion in the *drp-1* gene, a frozen *C. elegans* deletion library previously constructed (Harold Hutter and Barbara Conradt, unpublished) was screened through PCR using primers flanking the locus. One deletion sample was identified, and selections were performed to isolate the homozygous deletion strains. However, a homozygous *drp-1* mutant could not be isolated due to both sterility and embryonic lethality most likely caused by the *drp-1* deletion. A heterozygous *drp-1(bc259)* strain was isolated through PCR, 2 times out crossed with N2, and then balanced with the strain *MT1147 (dpy-13(e184sd) unc-8(n491sd)).* The deletion starts at aggtggacaaa and ends at tgaacgcc in the *drp-1* locus T12E12.4a cutting through exons 2 and 3 leading to a frameshift stop codon 50 bp upstream in exon 3. To quantify the effects of *drp-1(bc259)*: 1) The percentage of embryonic lethal and sterile progeny was determined by dividing the number of progeny displaying these phenotypes over the total number of progeny laid from *drp-1(bc259)/+* animals. 2) Mitochondrial morphology was determined by transferring *drp-1(bc259)/+* L4 or young adults onto NGM plates containing 30 μm rhodamine B (see section 5.5 and 5.6). 3) Whether *drp-1(bc259)* homozygous animals had a defect in programmed cell death was determined by counting cells in the anterior half of the pharynx of *drp-1(bc259)* animals that escaped the embryonic lethality (cell counting was performed by Barbara Conradt).

5.5 Mitochondrial staining and optimizing

Various concentrations of various mitochondrial specific voltage-sensing fluorescent dyes were tested to achieve optimal staining of mitochondria within embryos. Since embryos are protected by chitin egg shell, embryos could not be labeled directly. Mitochondrial dyes were transmitted through the germline of hermaphrodite mothers. Of importance, all experiments were performed the day after L4 or young adults were transferred to plates, containing the voltage-sensing dye, since this achieved best results.

L4 or young adults were transferred to NGM plates containing 30 μm rhodamine B hexyl ester perchlorate (rhodhamine), 30 μm tetramethylrhodamine ethyl ester perchlorate (TMRE) or 30 μm MitoTracker Red (all dyes obtained from Molecular Probes). It was found that both rhodamine and TMRE gave excellent staining, while the mitotracker signal was both weaker and less stable after the start of data acquisition. Both rhodhamine and TMRE were used for further experiments. Rhodamine, to define the mitochondrial structure within embryos and TMRE to observe changes in the mitochondrial membrane potential

5.6 Imaging of mitochondria

To reveal mitochondria in embryos, GFP fused to a sequence targeting the mitochondrial matrix (mitoGFP) was expressed in embryos (see section 2.1.1 and 2.1.2 for description), and/or staining with potential-sensitive mitochondria-selective rhodamine derivatives, rhodhamine or TMRE, was performed. Embryos were mounted on slides with agar pads (2%) and M9 buffer. Mitochondrial morphology was analyzed by confocal microscopy using either a Leica TCS SP2 (Leica Lasertechnik, Bensheim, Germany) or a Zeiss LSM 510 (Carl Zeiss Microscopy, Jena, Germany) equipped with a 63 × objective and DIC settings. Image acquisition was performed at ambient temperature (about 22 °C). For time-lapse imaging, 512 × 512 pixel images at 0.5-µm focal increments were recorded every 5 min, simultaneously imaging mitoGFP, rhodamine and DIC over a period of 30−60 min. Images were processed with the Leica Confocal Software (LCS) or LCS Lite. For all other imaging, 512 × 512 pixel images of single confocal planes were acquired simultaneously imaging mitoGFP, rhodamine and DIC and processed with Leica Confocal Software (LCS), LCS Lite or Bitplane Imaris 4.

For imaging the expression of transgene combinations, P_{hs}*mitogfp* and P_{hs}*egl-1,* P_{hs}*mitogfp* and P_{hs}*drp-1*(*K40A*), and P_{hs}*mitogfp* and P_{hs}*drp-1*, heat shock was applied as follows: at least 10 gravid transgenic hermaphrodites were transferred to fresh plates, allowed to lay eggs for 1 hr at 20ºC, heat-shocked for 45 min at 33ºC, and then allowed to recover for different time periods (specified in sections 2.2.2, 2.3.3, and 2.4.2 respectively) at 20ºC before being removed from the plates at the time imaging was performed.

5.7 Bacteria mediated *drp-1* RNAi

The *drp-1* or *gfp* containing pL4440 plasmid serving as feeding vectors were transformed into *Escherichia coli* HT115 cells. Single colonies were streaked onto LB/Amp plates. A streak of the bacteria was briefly mixed with 100 µl of LB to seed 3.5 cm NGM plates additionally containing 50 µg/ml Amp, 12.5 µg/ml tetracycline and 6 mM IPTG. To synchronize the animals, at least 6 L4 larvae were transferred to fresh cultivated plates containing specific dsRNA expressing bacteria per experiment. The next day, the now young adults were transferred to freshly seeded (12 hours prior) dsRNA expressing bacteria. Embryos were examined for survival where the young adults were exposed for 24 h to 48 h to the bacteria in both wild-type and *rrf-3(pk1426)* animals. The *rrf-3(pk1426)* animals were shown to be more sensitive towards bacteria mediated RNAi, especially in neuronal cells (Simmer et al., 2002). To quantify the effects of *drp-1*RNAi silencing the following tests were performed: 1) The percentage of embryonic lethality was determined by dividing the number of progeny that had arrested over total number of progeny laid from adults that had been fed dsRNA expressing bacteria (as described above). 2) Defects in programmed cell death were determined by both counting the number of cell corpses within the embryo and by counting the persistence of "extra cells" in the anterior half of the pharynx (counted by Barbara Conradt) of progeny laid from adults and there parents who had been fed dsRNA expressing bacteria. Cell corpse accumulation was done in embryos of *ced-1(e1735)* where cell corpse removal is blocked (Zhou et al., 2001).

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5.8 Cell death assays in transgenic animals

To quantify the programmed cell death enhancement of overexpressing transgene combination, P_{hs}*mitogfp* and P_{hs}*drp-1*, heat shock was applied as follows: at least 10 gravid transgenic hermaphrodites were transferred to fresh plates, allowed to lay eggs for 1 hour at 20ºC, heat-shocked for 45 min at 33ºC, allowed to recover for a 1.5-2 hour period before being removed from the plates and analyzed for the number of refractile corpses in embryos measured by DIC.

To quantify the programmed cell death defect of animals expressing the transgene P_{egl-1}*mitogfp* the "extra cells" in the anterior pharynx were counted by DIC in transgenic, non-Unc L4 larvae (performed by Barbara Conradt). To quantify the programmed cell death defect of overexpressing transgene combinations, P_{hs}*mitogfp* and P_{hs}*drp-1*(*K40A*) or P_{hs}*drp-1*(*K40A*) alone, heat shock was applied as follows: at least 10 gravid transgenic hermaphrodites were transferred to fresh plates, allowed to lay eggs less than an 1 hour at 20ºC, heat-shocked for 45 min at 33ºC, briefly allowed to recover, adults were removed from plates to ensure that all embryos on plates had been heat shocked, and then later after development proceeded "extra cells" in the anterior pharynx were counted by DIC in transgenic, non-Unc L4 larvae (performed by Barbara Conradt).

To quantify the embryonic lethality of expressing transgene combinations, P_{hs}*mitogfp* and P_{hs}*drp-1*(*K40A*) and P_{hs}*mitogfp* and P_{hs}*drp-1*, heat shock was applied as follows: at least 10 gravid transgenic hermaphrodites were transferred to fresh plates, allowed to lay eggs 1 hour at 20ºC, heat-shocked for 45 min at 33ºC, allowed to recover for 1 hour, adults were then removed from plates to ensure that all embryos on plates had been heat shocked. The percent transgenic animals were determined by counting the total number of adults and the number of non-Unc transgenic adults that developed from the heat shocked embryos. The percent embryonic lethality was determined by counting the total number of embryos treated and the number of embryos that failed to hatch within 24 hr of the treatment.

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6 List of Publications

Jagasia R, Grote P, Westermann B, Conradt B (2005) DRP-1-mediated mitochondrial fragmentation during EGL-1-induced cell death in C. elegans. Nature 433:754-760.

Fordyce, C., **Jagasia, R**. and Schlichter L.C. (2005) Microglia Kv1.3 channels control their ability to kill neurons. Journal of Neuroscience. In press. Ref # JN-RM-1251-05

7 Curriculum Vitae

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