Chapter V

- in which nematodes transgenic for wtHtt and muHtt are generated; expression of the wtHtt transgene shows no effect whereas expression of the muHtt transgene leads to increased developmentally regulated apoptosis

1 Introduction

In its natural environment Caenorhabditis elegans is a free-living nematode that thrives in the soil of the world’s temperate regions where it feeds on microorganisms. However, as with a lot of other animals, it was domesticated thanks to Sidney Brenner who first proposed in the mid-1960’s to study C. briggsae as a convenient model organism. In the laboratory, C. elegans is easy to maintain with bacteria as a food source, ambient temperature and atmospheric oxygen as the only growth requirements (Brenner, 1974).

C. elegans (Riddle et al., 1997) is a self-fertilizing hermaphrodite and produces both sperm and oocytes. Nonetheless, males occur at a rate of 0.1% and produce sperm, but no oocytes. To reproduce, males must mate with hermaphrodites, which leads to competition of male and hermaphrodite sperm. Male sperm usually outcompete hermaphrodite sperm resulting in cross-fertilization and the generation of 50% males in the offspring. Self-fertilization produces only hermaphrodites. Special laboratory techniques are able to generate females that only produce oocytes but no sperm. Until fertilized by a male, these animals lay unfertilized eggs that do not hatch. This sexual organization has made the nematode very useful for genetic analysis.

The lifespan of C. elegans at 20°C is ~15 days, which can be slowed or accelerated by maintaining the worm at, lowered or elevated temperature,
respectively. The life-cycle (Fig. 1) from egg to egg-producing adult is 3.5 days. Eggs are self-fertilized inside the uterus of the animal and start embryogenesis even before they are eventually laid, which occurs ~5 hours post-fertilization (Sulston et al., 1983). Embryogenesis usually takes ~14 hours and produces more than half of the cells of the adult animal. After hatching, fewer cells are produced anew, but instead they grow in size. Larval development (Sulston and White, 1980) is divided into four distinct stages (L1-L4), with successive moults at 29, 38, 47 and finally at 59 hours post-fertilization to produce the adult worm.

Growth of larvae can be arrested in a distinct L3 stage called dauer if growth conditions are adverse, e.g. lack of food and high population density. Dauer formation occurs before spermatogenesis and oogenesis take place, thereby utilizing the energy stored for these two processes to survive until conditions become better. Interestingly, animals with mutations in genes regulating dauer formation, e.g. daf-2 or daf-16 (Kimura et al., 1997; Lin et al., 1997), exhibit defects in the insulin/insulin-like growth factor (IGF)-signaling pathway that regulates glucose uptake and homeostasis. These mutations double the lifespan of the nematode. The dauer can survive for several months until conditions get more pleasant. It then moults into L4 and development continues normally.

The fate of every single cell in *C. elegans* was determined and complete cell lineage descriptions were obtained (Sulston and Horvitz, 1977; Sulston et al., 1983). *C. elegans* is the only multi-cellular organism in which such a feat has been accomplished. During development 1090 cells are generated in the hermaphrodite, 1179 in the male. Of these cells 131, die by intrinsic apoptosis in the hermaphrodite, in the male this number is 148 (Table 1).
In addition to the known fate of every cell, every single base of DNA is also known. The 97Mb genome is distributed on one X-chromosome and 5 autosomes which are fully sequenced (Consortium, 1998). It has been estimated that it encodes ~ 19,000 genes. Many of the known genes are evolutionarily conserved and have mammalian counterparts, many of which have only been identified by sequence homologies to the *C. elegans* genes. Therefore, *C. elegans* represents a useful tool for biological disciplines as diverse as molecular biology, genetics, evolution, even development and behaviour. As such, it allows parallels to be drawn to higher organisms, including humans.

As described earlier, the *C. elegans* death machinery provided the general blueprint for apoptosis (Horvitz, 1999). Based on findings in the nematode, corresponding counterparts in mammals have been identified. In fact, for the longest time, the Apaf-1 protein family was regarded as the CED-4 family, since mammalian homologues remained elusive. The *C. elegans* core death machinery, an analogue to the mammalian apoptosome, is decidedly simple (Metzstein et al., 1998). It consists of CED-9 (Hengartner and Horvitz, 1994), the analogue to anti-apoptotic Bcl-2 proteins, CED-4 (Yuan and Horvitz, 1992) and CED-3 (Yuan et al., 1993), the analogue to caspase-9. CED-4 differs slightly from Apaf-1 since it does not contain WD40 repeats. The possibility cannot be excluded that there exists a more perfect mammalian analogue to CED-4, but it has not been identified. These structural differences account for the mechanistic differences of apoptotic execution. In mammals, the WD40 domain of Apaf-1 binds cytochrome c, which leads to Apaf-1 oligomerization and formation of the apoptosome. In the worm, there is no cytochrome c requirement and CED-4 is kept from activating CED-3 by binding to CED-9. CED-9 in turn
is negatively regulated by the BH3-only protein Egl-1 (Chaudhary et al., 1998; Chinnaiyan et al., 1997; Chinnaiyan et al., 1997; Conradt and Horvitz, 1998; Wu et al., 1997; Yang et al., 1998).

Interestingly, database searches did not reveal a nematode homologue for Htt. This indicates that Htt evolved as part of higher organisms and therefore seems to perform a function that is restricted to them, possibly a role in the more complex nervous system. Drosophila and zebrafish Htt homologues exist, as well as mouse, rat and human versions. However, the drosophila Htt protein is the least conserved.

In previous chapters, caspase-9 was identified as the molecular target of Htt function. Consequences of this targeting included inhibition of cytochrome c-dependent caspase-9 activity and processing, as well as disruption of apoptosome formation. Since caspase-9 and CED-3 are close relatives, it is conceivable that Htt might also perform similar actions in the nematode. Htt binds the catalytic domain of caspase-9, which could lead to inhibition of intrinsic catalytic activity of the zymogen. Secondly, the analogue to disruption of apoptosome formation might also take place in the nematode, so that CED-3 will not bind to CED-4 due to inhibition by Htt. However, it is important to keep in mind that the nematode ‘apoptosome’ functions differently from the mammalian apoptosome and is already assembled, instead of induced to assemble after cytochrome c binding. Notwithstanding these caveats, C. elegans is probably the most convenient Htt +/- background and allows to assess Htt effects on a evolutionarily conserved signaling pathway in a healthy adult animal. Therefore, the wtHtt construct used in the previous chapters will be used to generate a transgenic nematode.
In addition, the effects of muHtt can easily be assessed in the worm. Two reports were published a short time after these studies were begun that addressed the question of poly-Q overexpression in the worm. In one study, a green fluorescent protein (GFP)-poly-Q fusion protein was introduced into C. elegans and was found to form aggregates and to activate the heat shock response (Satyal et al., 2000). Additionally, development was slowed. These effects were inhibited if HSP were introduced, indicating that protein misfolding was responsible for the observed effects. In the second study (Faber et al., 1999), a short N-terminal Htt fragment, corresponding to Exon1, was specifically expressed in a subset of neurons and increased susceptibility to cell death of these neurons that was dependent on CED-3 function. These studies, however, fail to address the question of the context of the larger protein. In addition, neither of these studies specifically takes advantage of the countless worm strains that have mutations in apoptotic effector molecules and therefore fail to provide a mechanistic basis for their findings.

Thus, in parallel experiments, both wtHtt and muHtt construct used in the previous experiments, will be introduced into C. elegans and the effect on the 131 programmed cell deaths will be assessed. In addition, several mutant worm strains with mutations in apoptotic effector molecules will be used to sensitize the assay conditions and to gain insight into the mechanisms of wt/muHtt function.
2 Results

2.1 Htt interacts with components of the C. elegans death machinery

To test whether experiments to assess the function of Htt in C. elegans were feasible at all, Htt constructs were overexpressed in 293 cells together with components of the C. elegans death machinery. After immunoprecipitation of the CED molecules, interaction was determined by Western blot for Htt (Fig. 2A). As shown, wtHtt interacted strongly with CED-3 and less so with CED-4. No interaction was observed with CED-9. These results are reminiscent of the experiments with the mammalian counterparts. Htt interacts with caspase-9, and with its analogue CED-3. It does not interact with Bcl-2 family members including CED-9. The only difference was the observed interaction with CED-4, which might be due to the structural differences between CED-4 and Apaf-1. The same interactions were observed when muHtt instead of wtHtt was used (Fig. 2B). These results demonstrated that C. elegans has the potential to serve as a useful model for Htt function.

2.2 Introduction of Htt constructs into wildtype C. elegans

In C. elegans, oocytes develop from precursor cells called distal tip cells (Riddle et al., 1997). During worm development, the distal tip cells migrate from their initial position near the vulva on the ventral side towards the ends of the animal, on to the dorsal side and back to the center of the animal. All the while, the distal tip cells produce immature oocytes which form a large syncytium on the dorsal side of the animal. Eventually, the oocytes in the syncytium mature and cell walls are being drawn in between individual cells.
The generation of transgenic nematodes proceeds slightly differently than the generation of transgenic mice. The plasmid containing the transgene is microinjected into the syncytium of L4 or young adult animals, where it reaches all cells that eventually form mature oocytes. Instead of being integrated into the genome, the transgene becomes incorporated as an extrachromosomal array, often containing several copies of the plasmid. Of the 200-300 F1 offspring that the worm is able to produce, ~10% carry the extrachromosomal array. Of these, another 10% stably integrate it into their germline and transmit to the F2 generation. To monitor integration, the plasmid is injected together with a marker plasmid, which usually is a phenotypic marker. The marker used here contains the rol-6 gene (Kramer et al., 1990), altering animal movement to a circular, rolling motion.

To obtain transgenic *C. elegans* lines for wt/muHtt, the N-terminal truncation mutants used in the previous chapters were subcloned into a vector containing the *C. elegans* heat shock promoter hsp16-2 (Fire et al., 1990). After injection into wild-type N2 worms, roller animals of the F2 generation were screened by PCR for the presence of the transgene (Fig. 3A). As shown, all roller lines were positive for the Htt transgene, indicating excellent correlation between the phenotypic marker and the presence of the extrachromosomal array. To confirm that the Htt transgene was expressed, worms were subjected to heat shock treatment and expression was subsequently analyzed by Western blot (Fig 3B). In worms that were not subjected to heat shock treatment, no Htt expression was observed, whereas robust upregulation was seen after one hour of heat shock. The detection limit for Htt was at least as low as twenty individual worms, demonstrating that Htt was expressed at very high levels.
Of the 131 programmed cell deaths occurring during *C. elegans* development, 16 occur in the pharynx (Fig. 4). Therefore, the pharynx represents a convenient area for assaying the effects of the Htt transgene. Adult animals were heat shocked for one hour and allowed to lay eggs for another hour to induce Htt expression in the developing embryo. The resulting offspring were scored in L3 stage by Normaski microscopy for changes in total numbers of pharyngeal cells. No differences with either wtHtt or muHtt were observed compared to wild type worms (data not shown).

2.3 Breeding of the Htt extrachromosomal arrays into different genetic backgrounds

Since no differences were observed in the N2 background, it was hypothesized that Htt interaction with the endogenous worm proteins was ineffective and therefore Htt function reduced. Thus, in order to improve sensitivity of the assay, the Htt worms were bred into different genetic backgrounds.

The CED-9 (n1950) mutation results in a gain-of-function and a complete suppression of all programmed cell deaths (Hengartner et al., 1992). This is caused by the inability of CED-9 (n1950) to bind Egl-1, which leads to constitutive activation of CED-9. Therefore, the CED-9 (n1950) animal contains additional pharyngeal cells, which can easily be counted.

Another mutation in the *C. elegans* effector machinery is CED-3 (n2877). This weak loss-of-function mutation (Shaham et al., 1999) partially suppresses apoptosis, which results in seven additional cells in the pharynx. While a weak Htt effect, maybe as little as +/- one cell, might go unnoticed
in the N2 background, this would statistically be more significant in the CED-3(n2877) background.

The last genetic background chosen to assess Htt function carries the CED-1(n1735) mutation (Zhou et al., 2001). These worms do not have any defect in the apoptotic effector machinery, but rather in the CED-1 transmembrane receptor protein, which leads to delayed recognition and subsequent clearance of apoptotic bodies from the organism. These apoptotic corpses are clearly recognizable by their button-like appearance and very easy to score (Fig. 4). In this background, the assay was performed slightly differently. While counting of apoptotic corpses was still restricted to the head area of the worm, animals were scored in early L1, to prevent progressive clearance of corpses. Analysis was not restricted to the pharynx, but also included the tissues around it.

In order to breed the Htt extrachromosomal array into these different genetic backgrounds, homozygous CED-9, CED-3 and CED-1 male lines were generated by simple breeding of the respective hermaphrodites with N2 males (Fig. 5). These males were then crossed with N2-Htt females. Females were generated by double-stranded RNA-mediated gene inactivation (RNA interference, RNAi) (Fire, 1999) of the FOG-3 gene. FOG-3 plays an important role in germ cell development and specifically leads to the generation of sperm (Chen et al., 2000; Ellis and Kimble, 1995). FOG-3 was inactivated in the germline by injection of dsRNA into the tail of L4 animals. The injected solution diffused freely throughout the worm, including immature germ cells and embryos. Once taken up by a cell, dsRNA inactivated the corresponding gene/mRNA by an unclear mechanism. Thus, all the resulting offspring had no sperm and was therefore female. The females were bred with the CED males and the presence of the
extrachromosomal array was monitored by the appearance of the roller phenotype.

2.4 Phenotypes

In order to express the Htt constructs during embryonal development, when most programmed cell deaths occur, adult animals were exposed to heat shock treatment for one hour. During this time, all fertilized embryos and unfertilized eggs were induced to upregulate Htt which lasted for about three hours after treatment. After heat shock the worms were allowed to lay eggs for an additional 1.5 hours. This ensured that only embryos and eggs that were exposed to heat shock were fertilized and thus expressed Htt during the crucial period of embryonic development. Since egg laying takes place five hours after fertilization, all eggs laid in this 1.5 hour period expressed Htt during the early parts of their development. With this regimen it was not possible, however, to influence the cell deaths that occurred after hatching (18 in the hermaphrodite, 37 in the male) since Htt expression was already downregulated.

For the CED-1 background, F1 animals were analyzed for the appearance of undigested apoptotic bodies after hatching when in early L1 stage. Animals were age matched by a gonadal primordium of four cells (the later distal tip cells). WtHtt and muHtt expressing animals were compared to control animals carrying the empty vector together with the rol-6 marker in the extrachromosomal array (Fig. 6). As shown, no difference was observed between control and wtHtt animals. In both lines, the same number of apoptotic corpses was observed (14.58 +/- 3.9, n=65 for ctrl, 14.43 +/- 3.5, n=55 for wtHtt, p=0.94). These numbers were somewhat smaller than reported for the CED-1 (n1735) mutation. However, the muHtt animals
showed a strong, statistically significant increase in apoptotic bodies of 22.25 +/- 6.9 (n=36, p<0.0001). This increase by about eight corpses clearly indicated that at least the mutant protein retained its function in the nematode and strongly induced apoptosis even in a non-mammalian background.

To confirm these results, the experiment was performed in the CED-3 background (Fig. 7). Here, animals were analyzed in the L3 stage to score additional pharyngeal cells unobstructed by muscle tissue. As in the CED-1 background, no difference was observed between control and wtHtt animals (34.96 +/- 4.1 for wtHtt, n=27). This further indicates that the anti-apoptotic function of wtHtt cannot be reproduced in the nematode. Nonetheless, muHtt expressing worms showed a statistically significant reduction in pharyngeal cells (31 +/- 4.9, n=23, p=0.031). This further confirmed the findings from the CED-1 background.

The experiments in the CED-9 background were only carried out in the preliminary stage. Thus, neither muHtt nor vector control animals were produced. The CED-9/wtHtt animals, however, showed a decrease in cell number above that of parental CED-9 (data not shown). These data suggest a pro-apoptotic function of wtHtt, which was clearly not observed in the experiments described in the previous chapters. It appears as if the CED-9 results are artifacts of this particular CED-9 mutation. The CED-9 (n1950) mutation disrupts binding of Egl-1 to CED-9, probably by altering the binding surface and thus the structure of CED-9. It is possible that this structural change also affects interaction with CED-4 and CED-3 and thus indirectly also with wtHtt. On the other hand, the experiments were performed using the parental CED-9 animals as a control and not the proper vector injected animals. Because of the peculiarities of the CED-9/wtHtt
phenotype, this line of inquiry was abandoned in favour of the more clear-cut CED-1 and CED-3 experiments.
3 Discussion

The worm model is an attractive system to study Htt function since no nematode homologue has been identified thus far. Any observed effect is therefore directly attributable to the expression of the transgene. A previous study addressed the question of muHtt function by expressing Exon1 in the ASH neurons of *C. elegans* and found sensitization to CED-3 mediated apoptosis of these cells (Faber et al., 1999). However, only Exon1 was expressed and only a subset of cells analyzed. This study therefore fails to address questions more directly related to HD, like the influence of the context of the larger protein and tissue specificity. Furthermore, since HD leads to apoptosis in select cells, this study neglects to investigate the effects exerted by expression of the Exon1 transgene on all programmed cell deaths in *C. elegans*.

Here, a large 548aa Htt fragment that contains at least two important regulatory motifs (caspase-3 cleavage site and Akt phosphorylation site) was introduced into the worm. Expression of the transgene is regulated by heat shock treatment. While the effect of the Htt is too weak to be observed in the wildtype N2 background, muHtt shows strong increase in developmentally regulated apoptosis in the CED-1 (n1735) and CED-3 (n2877) backgrounds. This is significant because Htt was also shown to interact with the two executioner molecules of the *C. elegans* death machinery, CED-3 and CED-4, in an overexpression system. Unfortunately, interaction of Htt with the endogenous proteins cannot be investigated, because the commercially available reagents are not sensitive enough. Interaction of muHtt with these core molecules of apoptosis apparently sensitizes some cells to apoptosis, that otherwise would not die. Alternatively, muHtt might also be able to
directly induce apoptosis in some cells; although it is difficult to explain why, if all cells express CED-3 and CED-4, only certain cells die.

The sensitization scenario is reminiscent of the mechanism proposed for muHtt function in mammalian cells (Chapter III): It was hypothesized that muHtt acts as a “Trojan Horse”, binding to caspase-9 and thereby activating caspase-3. The results observed in the nematode further stress this theory: MuHtt is able to interact with both, CED-4 and CED-3, thus either relieving CED-4 from inhibition by CED-9 or directly connecting CED-4 to CED-3, thereby activating it. Possibly, muHtt might also function to directly activate CED-3.

The *C. elegans* death machinery is probably the evolutionary precursor to the mammalian core death machinery. The key molecules in both cases bear strong similarities to their nematode/mammalian counterparts. It is very striking, that observations in these two fundamentally different experimental systems both support a single mechanism. While the observation in the ST14A system only weakly supported the proposed “Trojan Horse” idea, it gains more momentum by the results in the nematode system, which lead in the same direction.

However, this hypothesis needs further experimental confirmation. Currently, the CED-1/muHtt animals are being bred into loss-of-function backgrounds for CED-3, CED-4 and Egl-1. This will allow for investigation whether muHtt-induced apoptosis requires any of these molecules. Since the only reliably identified caspase in the *C. elegans* genome is CED-3, no muHtt-dependent apoptosis is expected be observed in the CED-3 (n718) loss-of-function background (unless muHtt-induced apoptosis utilizes another, as yet unidentified caspase; this finding in itself would be extremely significant). If the “Trojan Horse” hypothesis is correct and binding of
muHtt to CED-4 somehow activates CED-3, no apoptosis should be observed in the CED-4 (n1162) background, which carries an ochre mutation in codon 40 and therefore completely abolishes expression of CED-4. Because Egl-1 is transcriptionally regulated, pro-apoptotic transcriptional effects of muHtt can be assessed in the Egl-1 (n1084/n3082) loss-of-function mutation. With these tools at hand, the apoptotic mechanism of muHtt in *C. elegans* will be further unraveled.

In the past, observations of molecular mechanisms in *C. elegans* could be extrapolated to mammals, which were later confirmed. If a mechanism for muHtt-induced apoptosis can be found in *C. elegans*, this might also turn out to be the mechanism for muHtt-induced apoptosis in mammals, and thus the cause of HD. Using the nematode model, inhibitor studies can be performed to identify drugs to cure this devastating disease.

Interestingly, while the interaction profiles of muHtt and wtHtt are identical, wtHtt does not decrease rates of apoptosis in *C. elegans*. The observed pro-apoptotic effect of muHtt is therefore solely due to the expansion of the poly-Q stretch. These findings further confirm the gain-of-function model of HD.

Disappointingly, however, wtHtt does not inhibit developmentally regulated apoptosis in the nematode. Despite the similarities between the mammalian and the nematode death effectors, and even in the interaction properties of wtHtt, no protective effect is observed. Apparently, the different protein structures of CED-4 and Apaf-1, which lead to different caspase activation mechanisms, also influence the function of wtHtt. It was proposed in Chapter III that wtHtt inhibits proper formation of an active apoptosome. In the nematode, this functionality of wtHtt cannot be displayed as the nematode apoptosome is already pre-assembled and
needs to be released from CED-9 inhibition. Htt does not interact with CED-9 and therefore cannot influence this process (unless the results with the CED-9 (n1950) mutant are the result of changed interaction profiles, and wtHtt binds to CED-9 (n1950) and thus releases CED-4). The observed (non-) effects of wtHtt suggest that Htt does not inhibit catalytic activity of CED-3, but instead acts upstream of it. These observations are in support of the data gathered in the previous chapters in mammalian systems. It is important, though, not to overinterpret the available data.
4 Materials and Methods

4.1 Co-immunoprecipitation experiments

Co-immunoprecipitations with FLAG-CED-3, FLAG-CED-4 and AU1-CED-9 with the wtHtt and muHtt constructs were performed as described.

4.2 Plasmids and constructs

The vector pPDX was engineered by ligating an artificial multiple cloning site (5’-GATCCATA ATACGACTC ACTATAGGG AGA CCACGAATT CTGAGTCAC GAGACCCGG GTGAGTCAC GAGAGCGGC CGCATGAGT CACGAGAGGTAC-3’), containing an EcoRI, SmaI and NotI site, preceded by a T7 promoter site, into the BamHI/KpnI sites of the *C. elegans* heat-shock promoter containing vector pPD49.78. The pPDX-wtHtt and pPDX-muHtt constructs were generated by subcloning of the Htt constructs into the EcoRI/SmaI sites of pPDX. All constructs were verified by sequencing and *in vitro* transcription/translation as described earlier.

The rol-6 expressing vector pRF4 was used as a transformation marker, whose phenotype was followed through the generations.

4.3 Maintenance and transformation of C. elegans

*C. elegans* were routinely maintained on NGM plates with a freshly spread lawn of *E. coli* OP50 as a food source at 20°C. Breeding was done according to standard procedures. For heat shock experiments several animals were picked onto separate plates and exposed to 31°C for one hour. Animals were transferred off the plate after another 1.5 hours. Transformation of the nematode was achieved by microinjection of a
100ng/µl DNA mix of the desired pPDX vector and tenfold greater pRF4 into the syncytium of L4 animals. Worm lines with extrachromosomal arrays were maintained by selecting roller offspring for continued culture.

4.3 PCR analysis

Single worm PCR analysis was performed by lysis of individual animals in worm 5µl lysis buffer (10mM Tris-HCl, pH8.3, 50mM KCl, 2.5mM MgCl₂, 0.45% NP-40, 0.45% Tween-20, 0.01% gelatin), supplemented with 10mg/ml Proteinase K, by freezing in liquid N₂ for 20min, followed by incubation at 60°C for 2 hours and heat inactivation of the proteinase. Aliquots were then subjected to PCR using KlenTaq Advantage polymerase (Clontech).

4.4 Western blot analysis for Htt expression

After heat shock treatment, animals (rollers and non-rollers) from one almost food source depleted 10cm petri dish were collected, washed, resuspended in buffer A and flash frozen in liquid N₂. Frozen animals were ground to a fine powder in a pre-cooled mortar and centrifuged at 10,000xg for 10min. Equal amounts of total protein were analyzed by Western blot.

4.5 RNA-mediated interference

The FOG-3 cDNA was amplified by PCR from a C. elegans cDNA library with FOG-3 specific primers. Double-stranded RNA was then produced by performing in vitro transcription using the MEGAScript kit (Ambion) with the FOG-3 cDNA as a template. After purification on a p30 spin column (BioRad), dsRNA was injected into adult animals and female offspring used for breeding purposes.
4.6 Phenotypic analysis

Offspring of heat shocked CED-1 worms were age matched by the appearance of a gonadal primordium of four cells and scored for the number of apoptotic corpses. Similarly, CED-3 animals were scored in L3 stage for the number of pharyngeal non-muscle cells.
5 Figures and Legends

Figure 1

The lifecycle of *C. elegans*; the inner ring describes development from egg to adult animal; the outer ring describes the sexual maturation of the adult.
The lifecycle of *Caenorhabditis elegans* 
hours at 25°C
Table 1

Comparison of male and hermaphrodite/female cell fates.
hatching (800 mins)

<table>
<thead>
<tr>
<th></th>
<th>total cells made</th>
<th>number of programmed cell deaths</th>
<th>number of cells at hatching</th>
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<tbody>
<tr>
<td>Female</td>
<td>671</td>
<td>113</td>
<td>558</td>
</tr>
<tr>
<td>hermaphrodite</td>
<td>(17%)</td>
<td></td>
<td></td>
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<tr>
<td>Male</td>
<td>671</td>
<td>111</td>
<td>560</td>
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<tr>
<td></td>
<td>(16.5%)</td>
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adult (excluding germ line)

<table>
<thead>
<tr>
<th></th>
<th>total cells made</th>
<th>number of programmed cell deaths</th>
<th>number of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>1090</td>
<td>131</td>
<td>959</td>
</tr>
<tr>
<td>hermaphrodite</td>
<td>(12%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>1179</td>
<td>148</td>
<td>1031</td>
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<td></td>
<td>(12.5%)</td>
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**Figure 2**

HEK293 cells were transfected with wtHtt (A) and muHtt (B) constructs together with FLAG-CED3/-CED-4 and AU1-CED-9. The CED proteins were immunoprecipitated and Western blot was performed using α-Htt and α-FLAG/AU1 antibodies, respectively. Both, wtHtt and muHtt interacted with CED-3 and CED-4, but not CED-9.
Figure 3

(A) Single worm PCR analysis was performed on three stable roller/wtHtt lines, a non-roller and a roller control line. Vector pPDX-wtHtt was used as a positive control. As shown, the wtHtt fragment was only clearly amplified when Htt was present in the PCR reaction.

(B) One 10cm dish each of PCR-positive wtHtt-lines 3 and 4 was exposed to heat shock for 1 hour. Worms were harvested and lysed by grinding in liquid nitrogen and equal amounts of protein extracts were loaded onto SDS-PAGE. Western blot analysis with α-Htt antibody showed robust upregulation of the wtHtt transgene only in worms exposed to heat shock. The amount of protein loaded on the gel is the equivalent of ~20 worms. Line 4 showed stronger expression of the Htt band, consistent with the higher amount of segregation of the Htt-extrachromosomal array to the daughter generation observed in this line. Subsequently, line 4 was chosen for all further experiments due to the higher segregation ratios and higher wtHtt expression levels.
A

Htt fragment

N.S.

pPDX-wtHtt
line 1
line 3
line 4
non-roller ctrl line
roller ctrl line

B

line 3
- +
line 4
- +
heat shock

wtHtt
Figure 4

A wild-type (N2) worm in L2 stage (panel a): The two pharyngeal lobes are clearly visible. The distal tip cells have already left the four cell stage of the gonadal primordium and are migrating towards the anterior and posterior ends of the animal.

In the anterior pharynx, non-muscle pharyngeal cells, as seen by Normarski microscopy, stand out as flat, sponge-like depressions (panel b). Black arrows depict examples of ‘un-dead’ cells in the CED-3 (n2877) background in two different microscopic viewing levels.

Normaski microscopy of the head region of a CED-1 worm (panel c) clearly shows the button-like apoptotic corpses. Examples of corpses in different stages of disintegration are depicted by white arrows.
Figure 5

In order to breed the extrachromosomal Htt arrays into different genetic backgrounds, Htt females were generated by FOG-3 RNAi. The resulting females were bred with homozygous males carrying the desired CED mutation. Males were generated by crossing N2 males with CED hermaphrodites, followed by backcross of the resulting CED heterozygote males with CED hermaphrodites.
Breeding Scheme

FOG-3 RNAi

CED

Htt array

CED

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Figure 6

After exposure to heat shock for one hour, F1 CED-1/ctrl, CED-1/wtHtt and CED-1/muHtt animals were scored for the appearance of apoptotic corpses. No difference was observed between ctrl (white bars, 14.58 +/- 3.9, n=65) and wtHtt (black bars, 14.43 +/- 3.5, n=55). In contrast, muHtt expressing animals (gray bars, 22.25 +/- 6.9, n=36) showed a marked increase of apoptotic bodies.
number of corpses

number of animals

6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36
After heat shock, F1 CED-3/wtHtt and CED-3/muHtt animals were scored for the number of cells in the pharynx. MuHtt (black bars, 31 +/- 4.9, n=23) expressing animals showed a strong reduction of pharyngeal cells compared to wtHtt animals (white bars, 34.96 +/- 4.1, n=27), indicating an increased amount of cell death.
number of animals

number of pharyngeal cells
6 References


