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EOR-1/PLZF-regulated WAH-1/AIF sequentially promotes early and late stages of non-apoptotic corpse removal

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Abstract (159/180 words)

Programmed cell death (PCD) is a crucial genetically-encoded and evolutionarily-conserved process for development and homeostasis. We previously identified a genetically non-apoptotic, highly ordered, and stereotyped killing program called Compartmentalized Cell Elimination (CCE) in the *C. elegans* tail-spike epithelial cell (TSC). Here we identify the transcription factor EOR-1/PLZF as promoting CCE. Loss of EOR-1 results in a persisting un-engulfed large soma with enlarged nuclei. We find that EOR-1 and its partners positively regulate the transcription of the Apoptosis Inducing Factor AIF homolog, WAH-1/AIF. We report stereotyped and sequential spatiotemporal dynamics of WAH-1/AIF1 during phagocytosis, with

defined roles early and late. Mitochondrial to plasma membrane translocation is required for internalization, and plasma membrane to nuclear translocation for DNA degradation and ultimate corpse resolution. Our study expands our knowledge of PCD by describing a mechanistic contribution of EOR-1/PLZF and functional relevance to specific spatiotemporal contexts for WAH-1/AIF function, and by implying a correlation between DNA degradation with nuclear morphology during cell elimination.

Introduction

Programmed cell death (PCD) is a genetically programmed and evolutionarily conserved form of cell elimination vital for normal development and homeostasis (1). Defects can pose devastating consequences such as aberrant brain development and cancer therapy resistance(2-4). Apoptosis, arguably the most well studied form of PCD, is characterized by certain morphologic and genetic characteristics. Morphologically, apoptosis progresses by cell shrinkage, chromatin condensation, nuclear fragmentation, and mitochondrial fragmentation (1, 5, 6). Genetically, apoptosis is controlled by several conserved regulators including the cysteine protease caspase family. Caspases have a conserved pathway of regulators, including the pro-apoptotic Apaf-1, the antiapoptotic Bcl-2, and the proapoptotic BH-3 only in mammals (7-9). Recently, several forms of non-apoptotic regulated cell death have come to light (10) and their detailed mechanisms currently being elucidated, thereby adding to our knowledge of targets for treatment of human disease relevant to cell death.

In addition to the actual killing of cells fated to die, their remains must be efficiently removed via phagocytosis to avoid secondary necrosis and autoimmune consequences (11, 12). The mechanism of phagocytosis of apoptotic corpses has also been heavily studied, describing the molecular mechanisms of corpse engulfment, specifically in how dying cells are recognized (13) and how the phagocyte slated to be internalized extends around this corpse (14) and how, once fully internalized and encapsulated in a phagocytic vesicle called a phagosome, the corpse is eventually resolved through multiple steps of increased acidification of the phagosome (15) and introduction of lysosomal hydrolases (16). Apoptotic clearance is also well-known to occur via conserved pathways for engulfment and phagosome maturation following the complete internalization of the corpse (17, 18).

Much has been described in terms of cell killing and clearance at the molecular level using the powerful genetic model organism, the nematode *C. elegans* and the *C. elegans* germline (apoptosis) and male-specific linker cell (non-apoptotic LCD) have been informative settings to gain insights on PCD. Discoveries include the first description of the genetic regulation of apoptosis (19) and the discovery of a non-apoptotic form of cell death, Linker cell-type death (LCD) with conserved morphological features and genetic regulation (20). Phagocytosis has also been extensively characterized in the nematode, with seminal work done on corpse engulfment (21), the delineating of the molecular machinery and cell biology behind phagosome maturation (22). Newer studies include those describing roles in neuronal sculpting

and function (23), roles of LC3 lipidation in polar body removal (24) and multiple roles of the GTPase

RAB-35 in both apoptotic ell clearance and clearance following LCD (25, 26)

One regulator of both apoptotic and non-apoptotic (LCD) forms of PCD identified in *C. elegans* is

EOR-1, the ortholog the BTB/zinc-finger tumor suppressor transcription factor PLZF (27). In a non-cell

death context, EOR-1 is known to be involved in neuronal specification (28). In terms of PCD, EOR-

1/PLZF has been implicated both in apoptotic death of the HSN neurons (27) and the non-apoptotic

Linker-type Cell Death (LCD) (29). EOR-1 appears to play a specification role upstream to the execution

phase of these deaths acting upstream or in parallel to CED-9 in the HSNs and upstream of HSF-1 in

conjunction with WNT signaling in LCD (27). However, its precise mechanistic contribution to either form

of PCD remains unknown.

In addition to the core apoptotic gene regulation components, an additional regulator linked

specifically to germline apoptosis include the homolog of the mitochondrial flavoprotein, the Apoptosis

Inducing Factor AIF homolog, WAH-1(30, 31). Mammalian AIF is a multifunctional enzyme with essential

roles for both mitochondrial physiology and the elimination of dying cells (32). In *C. elegans*, WAH-1/AIF

has been associated separately with apoptotic cell removal at the level of both corpse recognition (30)

and DNA degradation (31).

We present here our application of an additional setting for PCD for specialized cells in *C. elegans*

in providing mechanistic insights into the genetic regulation and subcellular dynamics involved in different

steps of programmed cell elimination. We have previously described the tripartite cell killing program,

Compartmentalized Cell Elimination (CCE) of the scaffolding tail-spike epithelial cell (TSC) in the *C.*

elegans embryo, (33). Briefly, CCE entails three morphologically distinct cell elimination events that occur

in an ordered and morphologically stereotyped manner. The CCE program occurs 10 times slower than

most nematode cells fated to die apoptotically allowing for detailed examination of these events at the

gross and subcellar level (33). During CCE, the single process of the TSC segments into two

morphologically distinct sub-compartments that exhibit degenerative morphologies resembling two

different forms of neurite pruning (33). The soma appears to round, superficially resembling apoptotic

morphology (33). Owing to the distinct morphological profile of CCE, the demise of the TSC can serve as

a timekeeper for different events of cell elimination, both at the level of killing and clearance.

At the molecular level, CCE is genetically disparate from apoptosis (34-36). While it does require

the *C. elegans* caspase CED-3, it is not regulated by the homolog of BH3 only, the cell-specification factor

EGL-1/BH3-only (36). Multiple non-canonical regulators of CCE have been identified for cell killing (34-

36) In addition, we have found that the canonical engulfment machinery operates only on the soma

corpse (33), and that the process remnants require the cell-cell fusogen EFF-1 for autofusion of the newly

forming phagosome for phagosome closure (33). Thus, CCE is a promising system for generating new

insights into the molecular and cell biological machinery of programmed cell elimination.

In the present study, we leverage CCE as a morphologically and temporally well-defined, easily scorable, program to define a specific role of EOR-1/PLZF in the regulation of *wah-1*/Aif in promoting PCD. We describe defined spatiotemporal dynamics of WAH-1 as part of its dual sequential roles in cell corpse internalization and resolution. We also describe a corpse progression transition state seen upon failure of EOR-1/PLZF and WAH-1/AIF function. Our molecular and cell biological findings are highly relevant for neurodegeneration, metabolic disease and cancer biology studies and our work presents CCE as a viable setting to study new facets of the genetics and cell biology of programmed cell elimination.

Results

The transcription factor EOR-1/PLZF promotes CCE compartment-specifically

We have previously described a highly ordered and organized tripartite developmental cell death program, Compartmentalized Cell Elimination (CCE), in the nematode *C. elegans* (33). We have observed CCE in two polarized cell types in the embryo, the scaffolding tail-spike epithelial cell (TSC) and the sex-specific CEM sensory neurons(33). During CCE, these cells show three disparate regressive morphologies. Both the TSC **(Figure 1A**) and the hermaphrodite CEMs extend a single process/dendrite when intact (IMA, intact, mature). During early CCE (BA, beading, attached; BD, beading, detached), the single process/dendrite shows axon pruning-like morphology proximally and axon retraction-like pruning distally (**Figure 1B**). At this time, the soma rounds in a manner superficially resembling apoptotic cell death. Later in CCE, the distal process retracts into a distal node (**Figure 1C**) (soma, distal retraction, SDR) leaving behind a soma and a distal remnant (soma, distal degrading, SDD) (**Figure 1D**) which are phagocytosed stochastically by different neighboring phagocytes before the animal hatches. The highly organized and stereotyped elimination sequence of CCE allows us to use the cell's general morphology as a time-keeper for events in this cell death program.

While CCE is dependent on the main *C. elegans* caspase CED-3 (33) it is genetically distinct from apoptosis as it does not require EGL-1/BH-3-only (36) CCE is also temporally distinct from apoptosis as it takes longer to complete. While most cells fated to die apoptotically in *C. elegans* live for only 30 minutes (37), the TSC lives for 5 hours (38) with CCE of the TSC taking 150 minutes (33). This longer time-scale positions CCE of the TSC as a promising platform to better understand the fundamental cell biology of programmed cell elimination in greater detail.

Bearing this in mind and leveraging the facile genetics of *C. elegans*, we performed a forward genetic screen for CCE defects of the TSC using a membrane marker strain for the TSC (TSC-myrGFP) we have previously employed (33). We obtained a mutant, *ns957,* bearing an inappropriately persisting and enlarged cell body only, but with no process **(Figure 1E)**. This CCE defect increased when animals were raised and scored at 25°C when compared to 20°C **(Figure 1 F)**.

Intrigued by a mutant where only soma elimination fails but where the process is successfully
eliminated, we examined the morphology of our mutant more carefully using Differential Interference
Contrast (DIC) microscopy. Apoptotic cell death has well-documented morphological characteristics that
are visible under DIC microscopy, such as pyknotic nuclei and refractility (39). However, we observed in
our *ns957* mutant enlarged and non-pyknotic nuclei, and a non-refractile cytoplasm (**Figure 1G-G”**).

Following Whole Genome Sequencing, genomic DNA rescue (**Figure 1 I**), and examination of
other alleles (**Figure 1J,K-K'**), we found that our screen mutant has a causative lesion of E570K in the
gene encoding the transcription factor EOR-1/PLZF (**Figure 1H**). We also found from cell-specific rescue
experiments that *eor-1* acts cell-autonomously in the TSC (**Figure 1L**) and that eor-1 is expressed in the
(**Figure 1 M**).

EOR-1/PLZF acts downstream of CED-3/Caspase, but with known regulatory partners

We proceeded to examine the precise role of EOR-1 in PCD using CCE of the TSC as our model.
In its known roles in cell fate, EOR-1 functions with known co-regulators of transcription. We first
examined for a role of EOR-2, which binds to the BTB domain of EOR-1 and promotes it function, (40)
by looking at *eor-2 (cs42)* mutants (**Figure 2A, B, C**) and found this to phenocopy *eor-1* mutants. EOR-
1/PLZF has also been reported to act as a pioneer transcription factor that functions in conjunction with
the MAU-2 cohesin loader, and the SWSN-1 SWI/SWNF chromatin remodeling complex member to
modify chromatin architecture and gene expression of genes required for the maturation of the post-
mitotic development of the HSN neurons (28). To determine whether EOR-1 functions with MAU-2 and
SWSN-1 to promote CCE, we examined *mau-2 (qm160)* **(Figure 2 A,D, E***)* and *swsn-1 (ku355)* **(Figure
2 A,F, G)** mutant larvae and found that they show persisting TSC somas that phenocopy the distinct
morphology of *eor-1* mutant animals. Therefore, EOR-1 functions in the new setting of CCE via its
previously identified partners in transcription.

We next asked whether EOR-1 functions upstream or downstream of CED-3/caspase and
whether it impacts TSC fate in a more general way. As in the case of apoptosis, CCE requires the main
C. elegans caspase CED-3, as well as it's upstream regulator CED-4/Apaf1(36). We generated a *ced-
4(n1162); eor-1(cs28)* double mutant and found an intact TSC phenotype and nuclear phenotype
characteristic of *ced-3(n717)* single mutants (N=10/10), suggesting that EOR-1 acts downstream of CED-
4 and CED-3 to promote CCE and likely does not affect TSC development **(Figure 2H-H', I-I').**

EOR-1/PLZF is important late in CCE for corpse internalization

We next asked what aspect of CCE EOR-1 regulates. Given the striking soma morphology of *eor-
1* mutants, we examined closely the soma morphology changes across the different CCE stages in both
wild-type and *eor-1 (cs28)* mutants using a combination of DIC optics and our fluorescent TSC membrane

reporter. We noted down features of the TSC soma at different CCE stages. Prior to CCE, when the cell

has matured, but is still intact (Intact mature stage, IMA) (**Figure 3A-B"**), the soma appears polygonal

with its nuclei appearing ovoid, flat and speckled with many small refractile bodies (**Figure 3B'-B"**). As

CCE sets in, the soma becomes round and the proximal process displays beading (Beading attached

stage, BA) (**Figure 3C-D"**), during which the nuclei appear round and spheroid (**Figure 3D'-D"**). Nuclear

contents retain a speckled appearance of refractile material.

As CCE progresses to the next stage (soma-distal process retracting stage, SDR) (**Figure 3E-**

F'), the junction between the rounded soma is detached from the beading proximal process (**Figure 3E**)

and the distal process starts to retract into a node. At this stage, nuclei are rounded with intranuclear

contents beginning to condense into more discrete areas (**Figure 3F'-F"**). The soma and distal process

are the removed by separate phagocytes. At this stage, the soma forms a classical refractile, button-like,

appearance as it is starting to be engulfed. After the SDR/retraction stage, the soma and process appear

superficially to be degrading (Soma-distal process degrading stage, SDD) (**Figure 3G-H"**). The soma at

this point appears non-refractile and vacuolized, with its nuclei appearing pyknotic and detached **(Figure**

3 H'-H"). Apoptosis and other forms of PCD are defined by subcellular hallmarks such as specific

changes in nuclear morphology and cell enlargement. We find that, during CCE, pyknotic nuclei appear

also to exhibit Brownian motion within the non-refractile cytoplasm **(Figure 3H'-H"**). The TSC membrane

appears diffuse at this stage and the membrane loses its discrete ring surrounding the cell. We examined

eor-1 (cs28) TSC in a similar manner and found that CCE progression appeared normal up until the

SDD/soma-distal process degrading stage, where the nuclei fail to condense and the membrane does

not lose its integrity (**Figure 3I-P"**).

To better characterize the soma persistence defect of *eor-1 (ok1127)* mutants, we compared the

mutant defect with that of mutants for caspase/cell killing, *ced-3(n717)* and engulfment, *ced-12 (ky149),*

and performed epistasis. We compared the soma-persistence defects of *ced-3 (n717)* (**Figure 4 A-A"**)

and *ced-12(ky149)* mutants (**Figure 4 B-B"**) and *eor-1*(**Figure 4 C-C"**).   We observed that the TSC soma

morphological features of *eor-1* were distinct from both *ced-3 (n717)* and *ced-12(ky149)* mutants

suggesting the cell is neither living nor engulfed. Moreover, a double mutant for *eor-1* and *ced-12 (ky149)*

(**Figure 4D -D"**) phenocopied the *eor-1 (ok1127)* single mutants, rather than the refractile corpse of *ced-*

12 (ky149) mutants. This supports the idea that the persisting soma of *eor-1* mutants has progressed

through the killing steps of CCE but has not been engulfed and is consistent with an arrest at the SDD

stage. We proceeded to examine whether the persisting corpse of *eor-1 (cs28)* mutants is internalized

by its neighboring phagocyte. We used a cytosolic reporter labelling the TSC soma's neighboring

phagocyte (*skn-1p*::mKate2) and found that the persisting soma *eor-1* **(Figure 4 E-G; Supplemental**

Movie S1) mutants remains outside the phagocyt*e (*N=10/12)*, suggesting failure of corpse

internalization.

EOR-1/PLZF positively regulates *wah-1*/Aif expression in the TSC

We next sought to identify the transcriptional target for EOR-1/PLZF in the context of CCE and examined published data obtained from a previous ChiP-Seq analysis study of EOR-1/PLZF transcriptional targets (41)*.* We caught note of the gene *wah-1* which encodes WAH-1, the homolog of a mitochondrial flavoprotein, the Apoptosis Inducing Factor AIF1 homolog, a multifunctional enzyme that is essential for mitochondrial bioenergetic function (32). Interestingly WAH-1 has been shown to function to promote apoptotic cell death in the *C. elegans* germline (31). Upon activation by CED-3/Caspase and EGL-1/BH3-only, WAH-1/AIF promotes apoptosis by through DNA degradation via the CPS-6 endonuclease (31). WAH-1/AIF has also been shown to aid in phosphatidylserine (PS) exposure by promoting SCRM-1 scramblase function (30) to facilitate apoptotic corpse recognition.

We tested the hypothesis that EOR-1/PLZF promotes CCE through the transcriptional activation of *wah-1*. First, we looked at a mutant for *wah-1* in the TSC-myrGFP background. We found *wah-1* (*gk5392*) mutants to phenocopy *eor-1* mutants (**Figure 5A, B**), and the double mutant not additive (**Figure 5B**). We also found from cell-specific rescue experiments that *wah-1* functions in the TSC (**Figure 5C**). We examined an endogenous reporter for *wah-1* (*wah-1* promoter driven WAH-1::GFP) at the intact mature (IMA) embryo stage in wild-type (**Figure 5D**), *eor- 1(cs28)* (**Figure 5E**), *mau- 2 (gm160)*(**Figure 5F**), and *swsn-1(os22)* (**Figure 5G**) mutants and found decreased levels of *wah-1* expression in mutants(**Figure 5H**). These data support the notion that WAH-1 promotes CCE by acting within the TSC and is transcriptionally regulated by EOR-1, MAU-2 and SWSN-1. This is supported by modENCODE data for *wah-1* identifying an EOR-1 binding sequence at the *wah-1* promoter(42). Further, *wah-1* contains an HTZ-1 histone binding site, further supporting the involvement of a chromatin remodeling complex in maintaining *wah-1* transcription as cohesin loaders require nucleosome-free loci to bind.

WAH-1 functions in corpse internalization

We first considered WAH-1's possible role at the plasma membrane, as the soma rounds, and presumably about to be engulfed. Prior studies have shown that WAH-1/AIF promotes apoptotic corpse recognition, and engulfment (30). We looked at CCE progression in *wah-1* (gk5392) (**Figure 6 A-H"**) mutants and found that the progression differed from wild-type, arresting, as in *eor-1* mutants, at the SDD stage (**Figure 6 G-H"**).

Next, we used a cytosolic reporter labelling the TSC soma's neighboring phagocyte (*skn-1*p::mKate2) and found that the persisting soma of *wah-1* mutants remains outside the phagocyte **(Figure 6I-K)** (N=11/13), also similar to *eor-1* mutants. We also noted that in both *eor-1* and *wah-1* mutants the phagocyte pseudopods are not formed (**Figure 4E-G, Supplemental Movie S1; Figure 6I-K;**

Supplemental Movie S2), suggesting failure in corpse recognition. This contrasts with from our previous

work showing that mutations for the phagosome sealing fusogen gene *eff-1* do have extended

pseudopods and can recognize the TSC process remnants, though the phagosome cannot close (33).

WAH-1 has been shown to promote SCRM-1 scramblase flipping of phosphatidyl serine from the

inner to the outer plasma membrane (30). We found that mutants for *scrm-1(-)* also displayed CCE

defects (**Figure 6 L, M)** akin to *eor-1* and *wah-1* mutants. These data suggest WAH-1 is important for

TSC soma corpse recognition during CCE in a manner dependent of SCRM-1 as seen in its apoptotic

role.

WAH-1 also functions in nuclear dismantling after corpse internalization

WAH-1 has been previously shown to be involved in DNA degradation and is reported to

translocate from the mitochondria to the nucleus (31). How this correlates to the role at the plasma

membrane has not been determined. We attempted to address this both genetically and cell biologically

in the context of TSC elimination. We first tested whether in the context of CCE WAH-1 indeed functions

in DNA degradation and how this may impact the exaggerated nuclear morphology of *wah-1* and *eor-1*

mutants. To this end, we attempted to separate WAH-1 function in corpse internalization versus its

possible nuclear role.

WAH-1 has been shown to promote DNA degradation during apoptosis by acting to enhance the

activity of the CPS-6/Endonuclease G nuclease (31, 43). We found that *cps-6* mutants also phenocopy

wah-1 and *eor-1* mutants for CCE (**Figure 7A, B).** Moreover, a *wah-1; cps-6* double mutant did not show

an increase in TSC defects (**Figure 7A**) suggesting these genes function in the same genetic pathway.

We also found that mutants for *nuc-1*, a lysosomal acid hydrolase that promotes late DNA degradation

(44, 45), also phenocopy *eor-1* and *wah-1* mutants (**Figure 7A, C)** supporting a role for WAH-1 in DNA

degradation. Interestingly, it has been reported that degradation of DNA of apoptotic cell corpses requires

contributions of both the dying and the engulfing cell(45). NUC-1 has been shown to be released from

the apoptotic corpse into the phagolysosome allowing for DNA degradation.

We next investigated whether *cps-6* mutants were arrested at a phagosome maturation stage.

We looked at whether the soma of *cps-6* mutants is internalized, which we would expect if the loss of

CPS-6 affects a post-engulfment stage as the phagosome-containing the internalized corpse matures.

Interestingly, unlike for *wah-1* and *eor-1*, the soma of *cps-6* mutants appears to be internalized (**Figure**

7D-F; Supplemental Movie S3) (N=9/10), suggesting that CPS-6/EndoG is not involved in WAH-1's

corpse internalization function and rather likely plays its role in nuclear degradation downstream of

internalization during phagosome maturation to resolve the internalized corpse.

To test which stage of phagosome maturation WAH-1 and CPS-6 may function in nuclear

disintegration, we looked at known markers the sequential steps of phagosome maturation. We looked

at phagosomal markers for early (GFP::RAB-5) (**Figure 7G-G"**), late (GFP::RAB-7) (**Figure 7H-H"**) and
phagolysosomes (LAAT-1::mCherry) (**Figure 7I-I"**)(33) in *cps-6* mutants and found that the phagosome
bearing the internalized corpse is decorated by LAAT-1, suggesting arrest at the phagolysosomal stage.
This is consistent with a role of NUC-1 in TSC DNA degradation and supports NUC-1 as being a
phagolysosomal substrate. This also suggests a defect in corpse degradation causes the dramatic
nuclear and cytoplasmic phenotypes in mutants.

Defined spatiotemporal dynamics suggest dual functions of WAH-1/AIF before and after corpse
internalization
Having identified WAH-1/AIF as a regulator of CCE at both the level of corpse internalization and
degradation, we next examined its localization pattern of across CCE stages (**Figure 8**). We first
examined TSC promoter-driven WAH-1::mCherry against a TSC promoter-driven mitochondrial matrix
marker (**Figure 8A-D**). When the cell is intact, WAH-1 appears to be mitochondrial (**Figure 8A**) and not
in the plasma membrane. However, as the beading (BA/BD) stage begins and the soma rounds and has
detached from the process mitochondria and WAH-1 both appear to be at or close to the plasma
membrane (**Figure 8 B, C**). Afterwards, at the SDD stage, WAH-1 is not seen at the plasma membrane
but can be seen in what appears to be fragmented mitochondria but is largely delocalized and cytoplasmic
(**Figure 8D**). To better understand this localization pattern, we examined TSC mitochondrial localization
against the TSC plasma membrane. Interestingly, we found mitochondria to be close to the plasma
membrane through most of CCE (**Figure 8E, F, G**), appearing fragmented at the SDD stage when the
plasma membrane integrity is lost (**Figure 8H**).
We next attempted to position the TSC soma during the SDD stage relative to its phagocyte to
further assess WAH-1's role. In order to use the distinct morphology of CCE steps to temporally trace the
status of the TSC soma as it progresses through CCE, we imaged our TSC reporter against the soma-
phagocyte reporter in wild-type embryos (**Figure 8I-W**). As expected, the TSC soma is seen outside the
phagocyte at the IMA stage (**Figure 8I-L**) We found that the TSC soma is internalized from what we
define as the BD (beading, detached) stage when the soma rounds and appears refractile (**Figure 8M-**
P) through the SDR (**Figure 8Q-T**) and SDD (**Figure 8U-X**) stages. We observe WAH-1 outside of
mitochondria at the SDD stage (**Figure 8 D** and **8U-X**) after being at the mitochondria and plasma
membrane earlier at the SDR (**Figure 8 C** and **8Q-T**) and BD (**Figure 8 B** and **8M-P**) stages. Together,
these data suggest that a physical association between mitochondria and the plasma membrane, that
may involve WAH-1, may be important early in phagocytosis and that exit of WAH-1 from mitochondria
may be important for corpse degradation and digestion after internalization.

Discussion

Here we describe defined subcellular events of a developmentally killed cell as it progresses
through the sequential steps of phagocytic clearance. Our work supports a model in which WAH-1/AIF
dual function, positively regulated by transcriptional control by EOR-1/PLZF, ensures clearance of the
TSC corpse as a feature of CCE. We show that WAH-1/AIF is localized sequentially at very specific
stages of CCE that promote different phagocytic events, corpse internalization and, shown for the first
time in this study, corpse resolution during phagosome maturation when the corpse is in the
phagolysosome. We propose that this two-step role of WAH-1/AIF allows for controlled cellular demise
of the TSC soma. We provide an expanded cell biological and genetic characterization of CCE in *C.*
elegans, by assigning a specific and previously unidentified role *in vivo* for EOR-1/PLZF in the regulation
of *wah-/Aif1*. Our study expands on our knowledge of the contribution of WAH-1 on PCD by providing
spatial and temporal contexts for its function in CCE. Additionally, we present previously unreported
persisting cell corpse features in *C. elegans* during the failure of EOR-1/PLZF and WAH-1/AIF function.
CCE events are compartment-specific with different factors regulating the demise and removal of different
subcellular compartments (33). We find that WAH-1 and EOR-1 are also compartment-specific regulators
that impact only the removal of the soma, which is consistent with a role in DNA degradation and nuclear
changes. Our study expands on prior studies linking EOR-1/PLZF to apoptotic (27) and non-apoptotic
(29) PCD by providing a precise mechanistic contribution of EOR-1/PLZF in the context the CED-
3/caspase dependent but genetically non-apoptotic CCE program and provide evidence supporting that
WAH-1/AIF is an *in vivo* target of EOR-1/PLZF. We also introduce here a new corpse transition state
during when the cell elimination program is compromised, and propose that, just as killing, programmed
cell degradation must occur in a controlled highly regulated manner, not just via catastrophic destruction.
We propose that by regulating when DNA degradation and nuclear destruction takes place the corpse
facilitates its own controlled degradation in response to the conditions of the phagolysosomal
pH/enzymatic microenvironment.
Additionally, WAH-1/AIF studies to our knowledge have been limited to the *C. elegans* germ cell
apoptosis. During apoptosis, WAH-1's mitochondrial origin (31) and role in corpse recognition (30) and
as a nuclease (31) have been described. Here we describe its role in a genetically non-apoptotic program
and capitalizes on the longer time scale of CCE to capture sequential spatiotemporal dynamics of WAH-
1 within the cell corpse as it progresses through phagocytosis stages and correlate the cell's
microenvironment with WAH-1's localization and function. Future studies on WAH-1 dynamics and
function for other forms of PCD such as LCD would be of great interest to the field. It will be interesting
to explore whether the same transcriptional relationship between EOR-1 and *wah-1* exist in these other
forms of PCD.
Notably, WAH-1 exit from mitochondria is reported to be dependent on EGL-1 (31). However,
CCE, while CED-3/caspase-dependent, is independent of EGL-1/BH3-only (36). The WAH-1 localization

pattern suggests WAH-1 can promote nuclear degradation independent of EGL-1, even though it is thought that WAH-1 may be translocated to the nucleus by EGL-1. The lack of EGL-1 involvement may affect WAH-1's ability to be released from mitochondria and may explain why WAH-1 is still associated to some degree with mitochondria until the SDD stage of CCE. The lack of EGL-1 may cause WAH-1's mitochondrial release to be markedly slower, which may explain why the time course of CCE is much longer than apoptotic germline cells. Of note, the TSC serves in tail scaffolding as the embryo develops (33) and must therefore persist until this it completes this function, making it one of the more long-lived cells programmed to die.

Our work also opens up several exciting questions for future exploration. It is known that WAH-1/AIF is an important mitochondrial factor(32), but it is unclear what guides its translocation to the plasma membrane and nucleus in a temporally regulated manner. Whether phosphatidyl serine is exposed during CCE is also yet to be addressed. Also of interest is the molecular mechanism by which WAH-1/AIF perceives the changing phagosomal environment and responds by translocating to the nucleus to execute its role in DNA and nuclear degradation. How does WAH-1/AIF know to translocate so specifically in the corpse at distinct phagosomal stages? We also suggest that failure to appropriately degrade DNA can lead to not only cell persistence but severe exaggerations in nuclear morphology. From our study, failure of DNA degradation whether the corpse is outside or inside the phagocyte leads to enlarged nuclear size. What is the intrinsic signal for this nuclear enlargement? How aberrant DNA stability translates to structural changes in the nucleus is also an interesting avenue to explore. Unexpectedly, we observed that the DIC and TSC membrane marker phenotypes of *eor-1*, *wah-1*, *scrm-1*, and *cps-6* mutants appear superficially similar (persistent enlarged soma with exaggerated nuclei), though we find only *cps-6* mutants to have internalized corpses. Further genetic screens may identify factors affecting nuclear shape and size that may resolve this surprising observation. Taken together, our study further solidifies CCE as a powerful system to offer new insights on the genetics and cell biology of programmed cell killing and clearance.

Materials and Methods

C. elegans **methods**

C. elegans strains were cultured using standard methods on *E. coli* OP50 and grown at 20ºC. Wild-type animals were the Bristol N2 subspecies. For most TSC experiments, one of two integrated reporters were used: *nsIs435=TSCp::myrGFP* or *nsIs685=TSCp::mKate2-PH.* In addition, *nsIs650=wah-1*p::WAH-1::GFP was used. Integration of extrachromosomal arrays was performed using UV and trioxsalen (T2137, Sigma). Animals were scored at 20ºC, unless otherwise mentioned.

Imaging

Images were collected on a Nikon TI2-E Inverted microscope using a CFI60 Plan Apochromat Lambda 60x Oil Immersion Objective Lens, N.A. 1.4 (Nikon) and a Yokogawa W1 Dual Cam Spinning Disk Confocal images were acquired using NIS-Elements Advanced Research Package. For still embryo imaging, embryos were anesthetized using 0.5 M sodium azide. Larvae were paralyzed with 10mM sodium azide. Widefield imaging was performed on a Carl Zeiss Axio Imager.M2 microscope with 63X oil immersion lens. Super-resolution images were taken using a VTiSIM Super resolution Live Cell Confocal Imaging System.

Quantifying CCE defects

TSC death defects were scored at the L1 stage. Animals were mounted on slides on 2% agarose-M9 pads, paralyzed with 10mM sodium azide, and examined on a Carl Zeiss Axio Imager.M2 microscope with 63X oil immersion lens. The persisting TSC was identified by fluorescence based on its location and morphology. At least 50 animals were scored per genotype. For images, each image is representative of 10 sampled.

Worm strains used in this study—

List gene alleles under different chromosomes linkage groups (LG I-X)

LGI: *ced-12(ky149), mau-2(qm160), scrm-1(tm698), cps-6(ok1718)*

LGII:

LGIII*: ced-4(n1162), wah-1 (gk5392)*

LGIV: *eor-1(ok1127), eor-1(cs28), ced-3(n717)*

LGV: *swsn-1(os22)*

LGX: *eor-2 (cs42) nuc-1 (e1392)*

Plasmids and Transgenics

Plasmids were generated via Gibson cloning. Primer sequences and information on the construction of plasmids used in this study are provided in (Supplemental Table 1). The full list of transgenes is described in (Supplemental Table 2). The full length or fragment of the *aff-1* promoter was used to label the TSC.

Quantification of WAH-1::GFP fluorescence intensity

Images of the intact TSC in animals harboring *wah-1* promoter driven *wah-1*::GFP in WT, *eor-1(cs28), mau-2(qm160)*, and *swsn-1(os22)* were collected using confocal microscopy as stated above using the same settings across all images. Sum intensity projections of the TSC soma were generated by following

TSCp::mCherry in Fiji Image J software and GFP intensity was then calculated using this software. CTCF
was calculated and graphed using Graphpad. All data points represent the average of 10 animals.
Statistical significance was determined by Mann-Whitney U test for comparison between wild type and
mutant animals.

Statistics

Sample sizes and statistics were based on previous studies of CCE and the TSC(33). Independent
transgenic lines were treated as independent experiments. An unpaired two-tailed *t*-test was used for all
persisting TSC quantifications (GraphPad Prism). For all figures, mean ± standard error of the mean
(s.e.m.) is represented. P-values are indicated in (Supplemental Table 3)

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NR and PG designed the experiments and wrote the manuscript. NR, MW, KJ, RS, performed the
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Figure Legends:

Figure 1. EOR-1/PLZF promotes CCE cell-autonomously. (A-D) Stages of CCE**.** IMA, intact mature
cell; BD, beading, detached; SDR, soma-distal retracted; SDD, soma, distal degrading. n = 10 biologically
independent animals each with similar results. **(E)** Mutant *ns957* at L1 stage showing persisting TSC
soma. n = 10 biologically independent animals each with similar results **(F)** Quantification of *ns957* CCE
defects. Data are mean +/_ s.e.m. Statistics: Anova, see Supplementary Table 3 for individual P values.
G-G") Enlarged view of *ns957* persisting soma to show DIC view. **(I)** Gene structure of *eor-1*. **(J)**

Quantification of *eor-1* fosmid DNA rescue of *ns95*7, n=50. (**K**) Quantification CCE defects of other *eor-*

1 alleles, n=150. (**L, L'**) Quantification of CCE defects of additional *eor-1* mutant alleles. (**M**) *eor-1* TSC-

specific rescue, n=150. (**N**) *eor-1* expression, *eor-1* promoter-driven mKate2. n = 10 biologically

independent animals each with similar results**.** Scale bar, 5 μm. Data are mean +/ s.e.m. Statistics: Anova

and two-tailed unpaired student's t-test, see Supplementary Table 3for individual P values.

Figure 2. EOR-1/PLZF functions in CCE with its known partners but likely downstream of CED-

3/Caspase. (A) CCE defects across *eor-1* alleles and *eor-2*, *mau-2* and *swsn-1* mutants. Persisting TSC

soma phenotypes in (**B, C**) *eor-2(cs42),* (**D, E**) *mau-2 (qm160)* n = 10 biologically independent animals

each with similar results and (**F, G**) *swsn-1 (ku355)* n = 5 biologically independent animals each with

similar results. The TSC remains intact at L1 in both *ced-3 (n717)* (**H**) and *ced-4 (n1162)); eor-1(cs28)* (**I**)

n = 10 biologically independent animals each with similar results**.** Scale bar, 5 μm. n=150 for statistics.

Data are mean +/_ s.e.m Statistics: two-tailed unpaired student's t-test, see Supplementary Table 3 for

individual P values.

Figure 3. Loss of EOR-1/PLZF causes corpse arrest late in CCE progression. CCE of TSC

(membrane marker) in wild-type (**A-F"**) and *eor-1(cs28)* (**I-P"**) mutants showing progression of soma

elimination. Left-most panels show whole cell. (**A-B"**) IMA stage in wild-type. n = 10 biologically

independent animals each with similar results, (**C-D"**) BA stage in wild-type, n = 8 biologically

independent animals each with similar results, (**E-F"**) SDR stage in wild-type, n = 10 biologically

independent animals each with similar results, (**G-H"**) SDD stage in wild-type, n = 10 biologically

independent animals each with similar results. (**I-J"**) *eor-1 (cs28)* IMA n = 8 biologically independent

animals each with similar results, (**K-L"**) *eor-1 (cs28)* BA n = 6 biologically independent animals each with

similar results, (M-N") *eor-1 (cs28)* SDR n = 10 biologically independent animals each with similar results,

(**O-P"**) *eor-1 (cs28)* SDD n = 10 biologically independent animals each with similar results Scale bar, 5

μm.

Figure 4. The cell corpse of *eor-1* mutants is not internalized by the neighboring phagocyte.

Enlarged view of TSC soma at L1 in *ced-3(n717)* (**A-A"**), *ced-12(ky149)* (**B-B"**)**,** *eor-1(ok1127)* (C-C"),

eor-1; ced-12 (**D-D"**), (**E-G**) Test for TSC soma corpse (green) internalization for *eor-1 (cs28)* mutant L1

larvae by phagocyte (magenta) at different planes of the phagocyte (upper , middle, lower). n = 10

biologically independent animals each with similar results. Scale bar, 5 μm.

Figure 5.EOR-1/PLZF-1 positively regulates *wah-1*/Aif transcription to promote CCE. (A) *wah-*

1(allele) CCE defect of TSC at L1 n = 10 biologically independent animals each with similar results. (**B**)

Quantification of *wah-1* mutant defect compared to *eor-1* and *wah-1; eor-1* double mutant. (**C**) Graph of

TSC-specific rescue of *wah-1* mutant phenotype. Endogenous *wah-1* expression intensity in wild-type

(D), *eor-1* **(E)**, *mau-2* **(F)** and *swsn-1* **(G)** mutants. **(H)** Quantification of *wah-1* expression. n = 10

biologically independent animals each with similar results. Scale bar, 5 μm. Statistics: two-tailed unpaired

student's t-test for scoring and Mann Whittney U test for fluorescence intensity, see Supplementary Table

3 for individual P values.

Figure 6. The cell corpse of *wah-1* mutants is not internalized and may function at the plasma

membrane. (A-H") CCE progression in *wah-1(gk5392).* **(A-B")** IMA *wah-1 (gk5392)* n = 4 biologically

independent animals each with similar results**, (C-D")** BA *wah-1 (gk5392)* n = 5 biologically independent

animals each with similar results, (**E-F"**) SDR *wah-1 (gk5392)* n = 4 biologically independent animals

each with similar results, (**G-H"**) SDD *wah-1 (gk5392)* n = 3 biologically independent animals each with

similar results **(I-K)** TSC and soma-phagocyte dual marker strain in *wah-1* mutant L1 showing failure in

TSC corpse internalization. **(L)** L1 *scrm-1* mutant showing persisting TSC soma and exaggerated nuclei.

n = 10 biologically independent animals each with similar results. **(M)** Quantification of *scrm-1* mutant

defect. Scale bar, 5 μm. Statistics: two-tailed unpaired student's t-test, see Supplementary Table 3 for

individual P values.

Figure 7. WAH-1 may promote nuclear degradation of the TSC soma corpse at phagolysosomal

stage. (A) Quantification of *cps-6(ok1718)* and *nuc-1(e1382)* CCE defect of TSC (soma persistence) at

L1. **(B)** Images of these defects. **(D)** Test for soma corpse internalization for *cps-6(ok1718)* across

phagocyte planes (upper, middle lower)**.** Localization of phagocyte GFP::RAB-5 **(G-G")**, GFP**::**RAB-7 **(H-**

H") and LAAT-1:mCherry **(I'-I")** relative to TSC corpse of *cps-6(ok1718)* mutant at L1 stage. n = 10

biologically independent animals each with similar results. Scale bar, 5 μm. Statistics: two-tailed unpaired

student's t-test, see Supplementary Table 3 for individual P values.

Figure 8. WAH-1 exhibits defined spatiotemporal dynamics during CCE progression correlated

with its function. TSCp::WAH-1::mCherry and TSCp::mito::GFP localization during CCE IMA **(A)**, BA

(**B**), SDR (**C**) and SDD (**D**) stages in wild-type embryos. TSCp::mKate2-PH (membrane) and

TSCp::mito::GFP localization during CCE IMA **(E)**, BA (**F**), SDR (**G**) and SDD (**H**) stages in wild-type

embryos. Relative position of TSC soma versus soma phagocyte (upper, middle and lower phagocyte

planes) at different CCE stages, IMA (**I, J-L**), BD (**M, N-P**), SDR (**Q, R-T**) and SDD (**U, V-X**). n = 10

biologically independent animals each with similar results for A-D. Scale bar, 5 μm.

Figure 9. Model. EOR-1-regulated WAH-1 translocates to different TSC soma sub-compartments to

sequentially promote corpse internalization and resolution.

Supplemental Tables:

Supplemental Table 1. Plasmids used in this study.

Supplemental Table 2. Transgenes used in this study.

Supplemental Table 3. Statistics and P values.

Supplemental Movies:

Supplemental Movie S1. Test for TSC soma internalization of *eor-1 (cs28)* mutants. Green, TSC
membrane, Magenta, phagocyte cytosolic marker.

Supplemental Movie S2. Test for TSC soma internalization of *wah-1* mutants. Green, TSC
membrane, Magenta, phagocyte cytosolic marker.

Supplemental Movie S3. Test for TSC soma internalization of *cps-6* mutants. Green, TSC
membrane, Magenta, phagocyte cytosolic marker.

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643

Rather et. al, FIGURE 1

Time

A

% TSC Persistance

wt
eor-1 (ok1127)
eor-1 (cs28)
eor-2 (cs42)
mau-2 (qm160)
swsn-1 (ku355)

n=150

B Membrane DIC
MERGE
eor-2 (cs42)

C

D
mau-2 (qm160)

E

F
swsn-1 (ku355)

G

H Membrane
DIC MERGE
ced-3 (n717)

H' Membrane
ced-3 (n717)

I Membrane
DIC MERGE
ced-4 (n1162);
eor-1 (cs28)

I' Membrane
ced-4 (n1162); eor-1 (cs28)

Rather et. al, FIGURE 3

Rather et. al, FIGURE 4

ced-3(n717)-L1   *ced-12(ky149)*-L1   *eor-1 (ok1127)*-L1   *eor-1(ok1127); ced-12 (ky149)*-L1   *eor-1 (cs28)*-L1

A Membrane DIC MERGE *wah-1 (gk5392)*

B % TSC Persistance — wah-1(gk5392), eor-1 (cs28), wah-1(gk5392); eor-1 (cs28) — ns

C *wah-1(gk5392)* TSCpro *wah-1* — % TSC Persistance — Line 1 — Non-transgenic, Transgenic — *

H P*wah-1*::*wah-1*::GFP intensity — Corrected Total Cell Fluorescence (AU) — WT, eor-1 (cs28), mau-2 (qm160), swsn-1 (os22) — n=10 — ** *** ns

P*wah-1::wah-1*::GFP

D WT **E** eor-1 (cs28) **F** mau-2 (qm160) **G** swsn-1 (os22)

Intact
TSC

Soma Distal Retracting
TSC
Phagocyte

Soma Distal Degrading
TSC
Phagocyte