ccz-1 mediates the digestion of apoptotic corpses in *C. elegans*

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Summary

During development, the processes of cell division, differentiation and apoptosis must be precisely coordinated in order to maintain tissue homeostasis. The nematode *C. elegans* is a powerful model system in which to study cell death and its control. *C. elegans* apoptotic cells condense and form refractile corpses under differential interference contrast (DIC) microscopy. Activation of the GTPase CED-10 (Rac) in a neighbouring cell mediates the recognition and engulfment of the cell corpse. After inclusion of the engulfed corpse in a phagosome, different proteins are sequentially recruited onto this organelle to promote its acidification and fusion with lysosomes, leading to the enzymatic degradation of the cell corpse. We show that CCZ-1, a protein conserved from yeasts to humans, mediates the digestion of these apoptotic corpses. CCZ-1 seems to act in lysosome biogenesis and phagosome maturation by recruiting the GTPase RAB-7 over the phagosome.

Key words: Apoptosis, *C. elegans*, Phagosome maturation, *ccz-1*, Digestion pathway

Introduction

The development of *Caenorhabditis elegans* offers an invaluable opportunity to understand and characterize events taking place at the level of single cells. In early embryos, cells have a defined and precise contact map that ensures the correct fate specification via cell-cell inductive signals (Hutter and Schnabel, 1995). During developmental progression, cell death occurs in specific cells of its invariant lineage (Sulston et al., 1983) and in the germ line of the adult in response to DNA damage, genotoxic stress or bacterial infection (Gumienny et al., 1999; Gartner et al., 2000; Aballay and Ausubel, 2001). Apoptotic corpses in embryos are engulfed and digested by neighbouring cells. The *C. elegans* hermaphrodite gonad is composed of two U-shaped syncytia (Hubbard and Greenstein, 2000). Apoptotic germ cells cellularize away from the syncytium and generate refractile cell corpses under differential interference contrast (DIC) optics. Such corpses are engulfed and digested by the sheath cells, which encase the germ line (Gummienny et al., 1999).

Pathways that regulate corpse recognition and engulfment have been extensively characterized (for a review, see Kinchen and Hengartner, 2005), but few genes that are involved in the maturation of the apoptotic-corpse-containing phagosome have been identified. These genes have been ordered in a linear pathway that includes, among others, the HOPS complex and the GTPases RAB-5 and RAB-7 (Kinchen et al., 2008) (and see below). These GTPases act as 'molecular switches' that, in the inactive form, are bound to GDP and are located in the cytoplasm in complexes with RAB GDP-dissociation inhibitors (RabGDI) (Ullrich et al., 1994; Seabra and Wasmeier, 2004). Following activation of this pathway – in this case after engulfment of an apoptotic corpse – RabGDI releases the Rab GTPase. Free GDP-bound Rab GTPases can then bind to the membranous organelle and then recruit its own guanine nucleotide-exchange factor (GEF), resulting in the exchange of GDP for GTP and hence activation of the Rab protein (Ullrich et al., 1994).

These proteins are recruited sequentially onto the phagosome. Each protein needs the more upstream component to be charged, and is required for the next downstream protein to bind to the phagosome. The maturation process allows other vesicles, such as lysosomes, to bind and fuse to the phagosome, first acidifying and then enzymatically digesting the apoptotic corpse (Kinchen et al., 2008; Yu et al., 2008). This pathway is well conserved during evolution from worms to humans and its deregulation has been linked to human diseases such as Hermansky-Pudlak syndrome, an autosomal recessive disease that results from abnormalities in the intracellular transport of vesicles such as melanosomes, causing skin and hair hypopigmentation and reduced visual acuity. *HSP4*, one of the human genes associated with this disorder, is in fact the human homologue of *ccz-1* (Hoffman-Sommer et al., 2005). Failures in the digestion of apoptotic corpses also lead to chronic polyarthritis caused by DNA that escapes from degradation in macrophages. The accumulation of this DNA in joints induces arthritis (Kawane et al., 2006).

In the present report we describe CCZ-1 as a new element in the pathway that mediates the degradation of apoptotic corpses. CCZ-1 seems to act both at the level of the exchange of the GTPase RAB-5 with the GTPase RAB-7 by recruiting RAB-7 onto the phagosome and also during lysosome biogenesis.

Results and Discussion

In a screening for maternal-effect lethal mutations on chromosome V of *C. elegans*, we found that three alleles (named *t2070*, *t2129* and *t2170*) of the same gene caused a striking set of defects. The first phenotype observed, in oocytes and embryos laid by mothers

homozygous for any of these mutations, was an accumulation of large vesicle-like structures (Fig. 1A,B). These vesicles were more abundant in the cortex of the anterior cell (named AB) at the two-cell-stage embryo and its descendants than in the posterior cell (P1). The second phenotype was that the mitotic spindle of the blastomeres EMS and ABar did not rotate, unlike the wild type (Fig. 1C,D; supplementary material Table S1), producing an abnormal pattern of cell-cell contacts. As a consequence of this, the normal left-right asymmetry of these embryos was disrupted, as determined by the position of apoptosis in the cell lineage, and the embryos died owing to an abnormal morphogenesis. Embryos in which the spindle rotated properly acquired a normal cell fate and underwent normal morphogenesis, but arrested at the two- to three-fold stage of morphogenesis. The accumulation of vesicles was still present in the cells of those late-arrested embryos. To better understand the nature of these vesicles, we expressed a fusion of yolk protein to GFP (VIT-2–GFP) (Poteryaev et al., 2007) in the *t2129* mutant. The yolk appeared properly endocytosed in the *t2129* oocytes but accumulated in the large and peripheral granules (supplementary material Fig. S1). Although we cannot exclude other defects, the inability to metabolize the yolk reserve during development might explain the arrest of the mutant embryos. The lethality of these mutants reached 99.5% of the embryos. The remaining escapers arrested as L1 larvae.

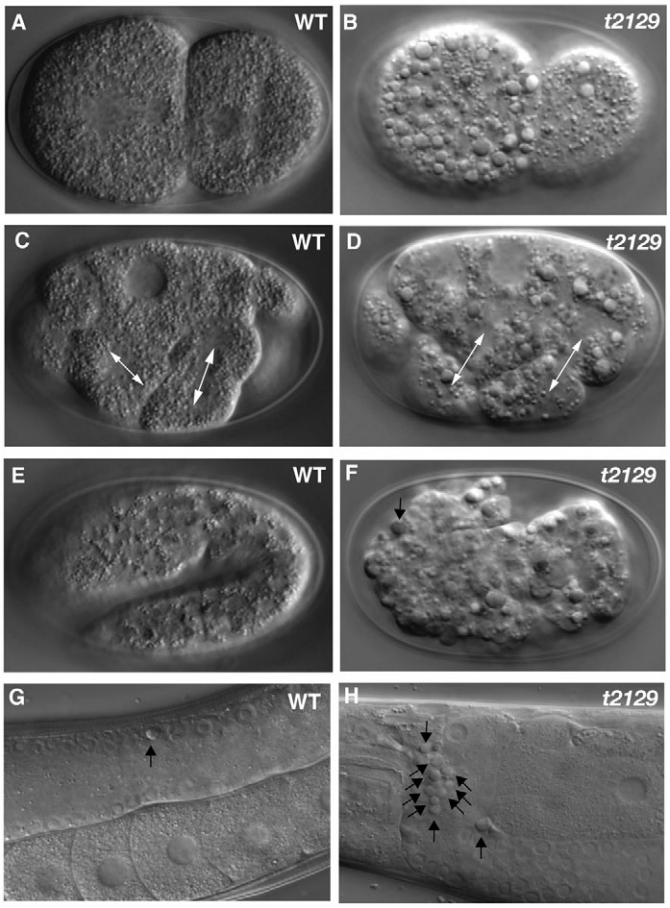

Fig. 1. Microscopic analysis of *ccz-1* mutants. Left column shows wild-type (WT) *C. elegans* at different stages: (**A**) two-cell stage, (**C**) 12-cell stage and (**E**) two-fold stage. Right column shows *ccz-1(t2129)* mutant embryos at the same stages as the corresponding left-hand WT panels. (**B**) *ccz-1(t2129)* homozygous embryos accumulate vesicles that can be observed with DIC microscopy. (**D**) The mitotic spindle of the blastomeres EMS and ABar does not rotate, unlike the WT. Double-headed arrows indicate orientation of the mitotic spindle. (**F**) *ccz-1(t2129)* mutant embryos at late stages still show an accumulation of vesicles as well as persistent undigested corpses. The black arrow shows an apoptotic corpse. See supplementary material Movie 1 for the explanation of how the embryonic corpses are detected. (**G**) In the WT hermaphrodite gonad, one or two corpses can be observed by DIC microscopy at 36 hours post-L4/adult molt. (**H**) However, in the homozygous *ccz-1(t2129)* hermaphrodites, a high accumulation of apoptotic cells is shown. Black arrows point to apoptotic corpses.

In addition, four-dimensional (4D) microscope analysis both of maternal and zygotic-defective *t2129*, *t2070* or *t2170* embryos revealed an accumulation of apoptotic corpses due to a defect in the clearance of corpses from apoptotic cells. A total of 25 focal planes of the embryos were recorded for 10 hours. As a result, 4D movies (three dimensions of the embryo + time) were obtained for each genotype. The SIMI Biocell software (SIMI GmbH, Germany) allowed each cell of the embryo to be traced in time and space, as well as tracing the mitosis and terminal differentiation of each cell. The cells placed in the lineage tree at positions in which the wild-type or the new ectopic fate corresponded to apoptosis were followed by 4D microscopy. These cells exhibited all the properties of a typical *C. elegans* apoptotic cell (asymmetric division, condensation, erythrocyte-like stage and refractile lentil stage), but remained as undigested persistent corpses (Fig. 1E,F; see also supplementary material Movie 1). To further confirm their identity as apoptotic cells, we generated a double mutant, *t2129*; *ced-3* (caspase) (allele *n717*). The absence of the only caspase-encoding gene in the *C. elegans* genome inactivated programmed cell death and no apoptosis took place in those embryos (supplementary material Table S1 and Movie 2). These results indicated that they really were apoptotic cells.

In *C. elegans*, the genetic pathways involved in apoptosis have been shown to play a role both in somatic cell death during development and germline apoptosis in adulthood (Kinchen and Hengartner, 2005). We then analyzed the gonad of the maternally rescued adult *t2070*, *t2129* and *t2170* mutant worms for persistent corpses. In the three mutants, we found a significant accumulation of persistent corpses, the number of which increased with time (Fig. 1H; supplementary material Fig. S2A) until they reached 22.9±6.34 (mean ± s.d.) corpses per gonadal arm 48 hours after the last molt, whereas a wild-type worm contained 1.75±2.22 corpses in the same conditions.

To better understand the state of these persistent corpses, we stained the worms with Acridine Orange (AO), a vital dye that selectively stains engulfed apoptotic cells within acidic organelles (Kinchen et al., 2005). Persistent corpses in the gonads of *t2070*, *t2129* and *t2170* mutant worms were AO positive, indicating that their accumulation is not due to a defect in the engulfment process, as has been described in *ced-1*, *ced-6*, *ced-5*, *ced-2*, *ced-12* or *ced-10* mutants (Kinchen and Hengartner, 2005). The AO staining suggested that, in these mutants, the corpses were efficiently engulfed and that they accumulated within phagosomes as undigested corpses (Fig. 2A). To further confirm this result, we performed transmission electron microscopy of the germline-persistent corpses in two *ccz-1(t2129)* mutant worms. As shown in Fig. 2B, five out of five analyzed corpses seemed to be totally engulfed within the sheath cells, indicating that the *t2129*, and by extension the *t2070* and *t2170*, mutations abolished digestion of apoptotic corpses, whereas earlier processes, such as dying itself or engulfment, remained unaffected. In addition, contrary to many

engulfment-defective mutants (Reddien and Horvitz, 2000), migration of the distal tip cells (DTCs) of the gonads from *t2070*, *t2129* and *t2170* maternally rescued homozygous worms was normal and the gonads developed the typical U-shaped structure of the wild type.

We identified the three mutations by using three-factor mapping, followed by single nucleotide polymorphism (SNP) mapping. These mutations define three alleles of the gene F58G11.6, hereafter referred to as *ccz-1* owing to its homology to the *Saccharomyces cerevisiae CCZ1* gene. The *t2070* mutation abolished splicing of the first intron, producing an abnormal mRNA (supplementary material Fig. S2B and Fig. S3A). *t2129* and *t2170* mutations result in premature STOP codons (supplementary material Fig. S2B and Fig. S3A,B). *t2070* and *t2129* both have a similar phenotype that is likely to be null. A knockout in *ccz-1* has recently become available (*ok2182*) from the *C. elegans* Gene Knockout Consortium. However, this deletion has an alternative splicing that restores a truncated but otherwise in-frame protein that retains some activity, as indicated by the viability of the homozygous mutant strain (supplementary material Fig. S3C,D). *ok2182* is therefore a hypomorph allele. RNA interference (RNAi)-mediated knockdown of *ccz-1* strikingly caused a similar weak phenotype, suggesting that either CCZ-1 is a very stable protein or that the double-stranded RNA (dsRNA) treatment was not very effective for this gene.

To further test whether these mutations were responsible for the phenotype, we performed a complementation experiment using plasmids containing a translation fusion of *ccz-1* to YFP under the control of the *ced-1* promoter, which drives the expression of the transgene in the engulfing cells, and another under the control of the endogenous *ccz-1* promoter. The embryonic lethality was rescued and the transgenic homozygous mutant worms cleared apoptotic corpses properly (supplementary material Fig. S4). These results indicate that loss of *ccz-1* function is responsible for the observed phenotypes in *C. elegans*: lethality, accumulation of vesicles, problems in spindle orientation and defects in the digestion of apoptotic corpses.

ccz-1 encodes a protein of 528 amino acids that is highly conserved from yeasts to mammals. The yeast protein has been characterized as being involved in a pathway for the fusion of vesicles to vacuoles or phagosomes (Sato et al., 2000; Wang et al., 2003). At the molecular level, the N-terminal part of CCZ-1 is predicted to have a ChiPS domain that might interact with GTPases (Kinch and Grishin, 2006). This N-terminal part is also structurally similar to SAND-1 and members of the TRAPPII complex, which is a tethering complex at the Golgi (Soding et al., 2005).

The yeast homologues of SAND-1 and CCZ-1 interact with each other, forming a complex that seems to link the HOPS complex to Ypt7p (the yeast orthologue of *C. elegans* GTPase RAB-7). Ccz1p-Sand1p, Ypt7p and the HOPS complex are sequentially recruited. Each protein mediates the loading of the following on the phagosome. Finally, recruitment of the HOPS complex enables interaction with surface proteins from other vesicles, allowing the fusion of their membranes to the phagosome

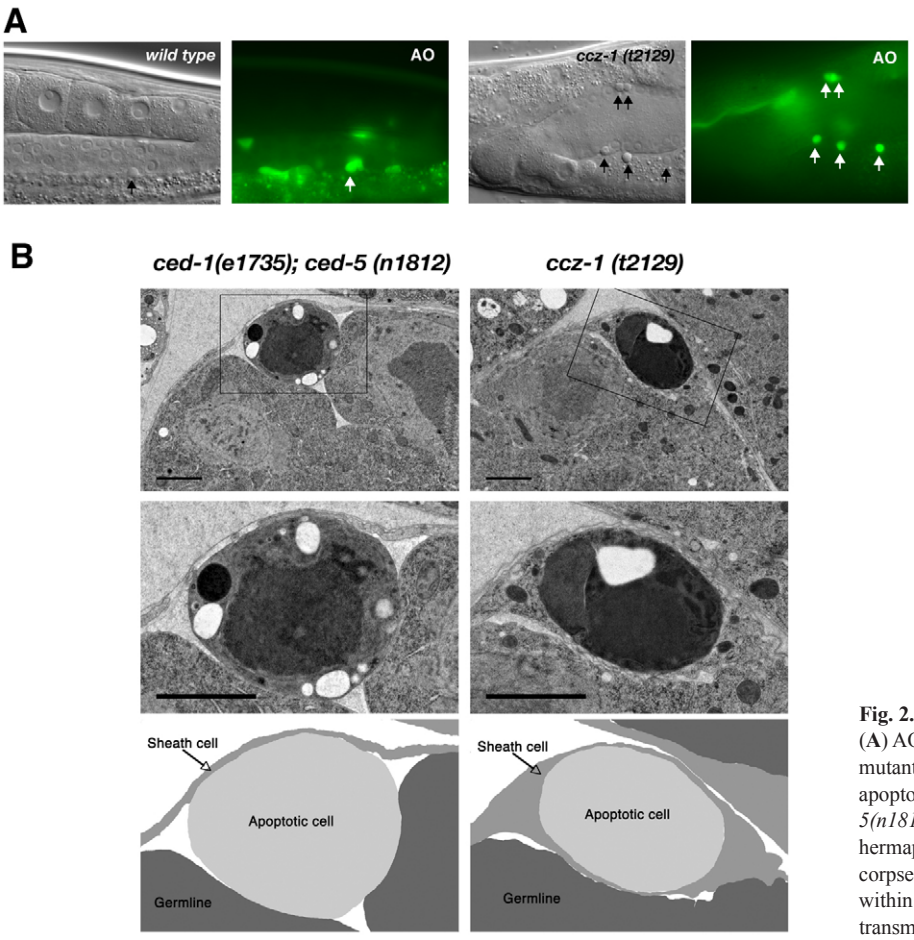

Fig. 2. Apoptotic cells are internalized in *ccz-1* mutants. (**A**) AO staining of wild type (left panels) and the *ccz-1* mutant apoptotic corpses (right panels). Arrows point to apoptotic corpses. (**B**) Double-mutant *ced-1(e1735); ced-5(n1812)* corpses are not engulfed by the sheath cells of the hermaphrodite adult gonad (left column), whereas cell corpses in the *ccz-1* mutant (right column) are internalized within the sheath cells (100%, *n*=5), as shown by transmission electron microscopy. Scale bars: 2 μm.

(Kucharczyk et al., 2001; Kinch and Grishin, 2006, Sato et al., 2000; Wang et al., 2003).

Similarly to yeast proteins, *C. elegans* SAND-1 and CCZ-1 can form a complex, as shown by their interaction in two-hybrid assays (Poteryaev et al., 2007). In addition, mutant worms defective in the GTPase *rab-7* or in proteins of the HOPS complex have recently been described as members of a pathway involved in the digestion of apoptotic cells whose inactivation leads to an accumulation of undigested corpses within phagosomes (Kinchen et al., 2008; Yu et al., 2008), similar to the defect we describe here for *ccz-1* mutants.

Because SAND-1 and CCZ-1 form a protein complex, we analyzed whether *sand-1* had a similar function to *ccz-1* in the digestion of apoptotic cells. Inactivation of *sand-1* indeed revealed the same three phenotypes that we detected in the *ccz-1* mutants: spindle orientation defects, embryonic corpses and germline corpses (supplementary material Table S1).

To further address the function of CCZ-1, we generated animals expressing fluorescent CCZ-1 protein. In the wild type, CCZ-1-tagged yellow fluorescent protein (CCZ-1–YFP) localized around refractile apoptotic corpses (Fig. 3Ba,Ba′). This localization was

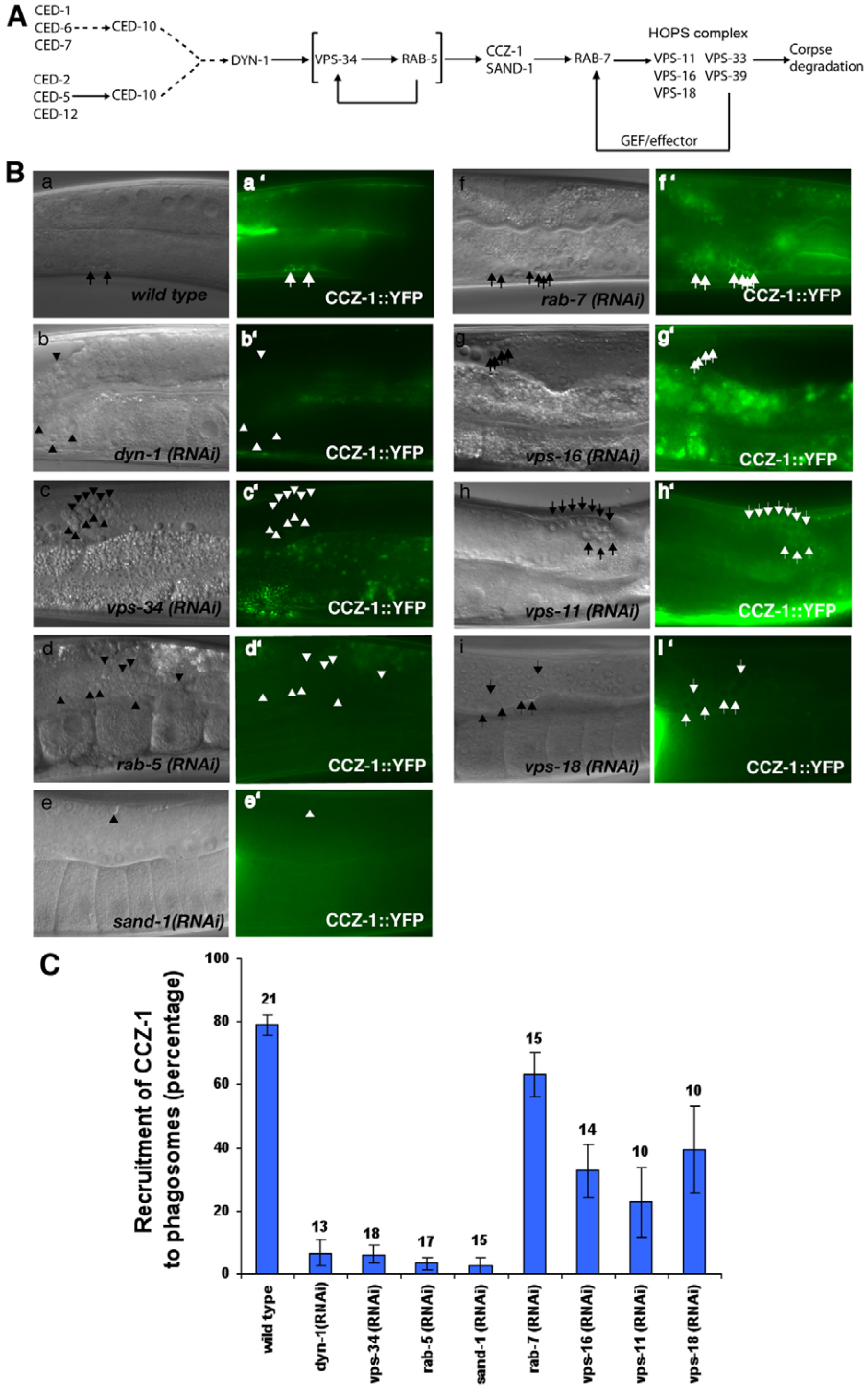

Fig. 3. Role for CCZ-1 in the phagosome-maturation pathway during the engulfment of apoptotic cells. (**A**) Genetic pathway: CCZ-1 acts downstream of RAB-5 GTPase and upstream of RAB-7 GTPase in the pathway for phagosome maturation. (**B**) CCZ-1 localization in the wild type and different mutants defective in the phagosome-maturation pathway. In the wild type (a,a′) CCZ-1–YFP forms halos around phagosomes containing engulfed apoptotic cells in the *C. elegans* gonad. In *dyn-1(RNAi)* (b,b′), *vps-34(RNAi)* (c,c′) and *rab-5(RNAi)* (d,d′) worms, these CCZ-1–YFP halos disappear, indicating that CCZ-1 acts downstream in the pathway. In *sand-1(RNAi)* mutants (e,e′), the correct localization of CCZ-1 is also blocked. In *rab-7(RNAi)* mutants (f-f′), the CCZ-1–YFP halos are visible, indicating that CCZ-1 acts upstream of RAB-7. In *vps-16(RNAi)* (g,g′), *vps-18(RNAi)* (i,i′) and in *vps-11(RNAi)* (h,h′) mutants, the CCZ-1–YFP halos are visible. Arrows indicate corpses positive for YFP halos; arrowheads indicate corpses negative for YFP halos. (**C**) Quantified data showing the recruitment of CCZ-1–YFP around the germline corpses. Apoptotic corpses were counted under DIC optics and scored for YFP-positive halos under ultraviolet light. The result was expressed in percentage. Numbers over the columns refer to the number of analyzed worms in each case. Columns indicate means ± s.e.m.

the same as that of RAB-5 and RAB-7 (Fig. 4) (Kinchen et al., 2008). In addition, the inactivation of *rab-7* by RNAi in a *ccz-1(t2129)* mutant worm produced an accumulation of apoptotic corpses in the gonad, similar to that produced by inactivation of *rab-7* alone (supplementary material Table S1). The defect caused by the inactivation of both genes was therefore not additive. These results (same localization of CCZ-1 and proteins of the 'corpse-digestion pathway', same phenotype and no additive effect in the double mutant) suggest that CCZ-1 and RAB-7 act on a single linear pathway involved in the digestion of apoptotic corpses within phagosomes.

To further determine whether CCZ-1 acts in the apoptotic-corpse-digestion pathway, and at which level, we tested the effect of its inactivation on the localization of the proteins in this pathway. We used transgenic worms expressing a YFP–2×FYVE construct, which has been shown to specifically bind phosphatidylinositol 3-phosphate [PtdIns(3)*P*], which is generated by VPS-34 on endosomes (Roggo et al., 2002) and recruited to phagosomes containing apoptotic corpses (Kinchen et al., 2008). The phagosomal recruitment of YFP–2×FYVE was not affected in the *ccz-1(t2129)* mutants, indicating that CCZ-1 acts downstream of VPS-34 (Fig. 4Aa-Ab′,B). Similarly, the YFP-tagged GTPase RAB-5 formed clear halos around the internalized corpses in the *ccz-1(t2129)* mutants (Fig. 4Ac-Ad′). However, we found a significant increase in the number of RAB-5–YFP-positive corpses in a *ccz-1(t2129)* background compared with a wild-type background (Fig. 4B). This increase suggests that CCZ-1 acts downstream of RAB-5 and that it could be involved in a mechanism aimed at removing RAB-5 from phagosomes during phagosome maturation.

By contrast, the recruitment of RAB-7–YFP around the apoptotic-corpse-containing phagosomes was dramatically reduced in a *ccz-1(t2129)* mutant as compared with the wild type (Fig. 4Ae-Af′,B), indicating that CCZ-1 acts upstream of the GTPase RAB-7.

Our results suggest a model in which CCZ-1 and SAND-1 would participate in the exchange of the GTPase RAB-5 for RAB-7 during the maturation of phagosomes. Therefore, CCZ-1 would act downstream of the GTPase RAB-5 and upstream of the GTPase RAB-7, and would be required for efficient removal of RAB-5 from the surface of the phagosome and RAB-7 recruitment during digestion of apoptotic corpses (Fig. 3A). Indeed, inactivation of any of these genes leads to the accumulation of undigested corpses. If this is the case, the absence of elements of the pathway upstream of RAB-7 would abolish the recruitment of CCZ-1 onto phagosomes, whereas inactivation of RAB-7 or elements of the HOPS complex would lead to a normal recruitment of CCZ-1 onto apoptotic-corpse-containing phagosomes. To further test whether this was the case, we used transgenic worms expressing CCZ-1–YFP under the control of the *ced-1* promoter. These animals were

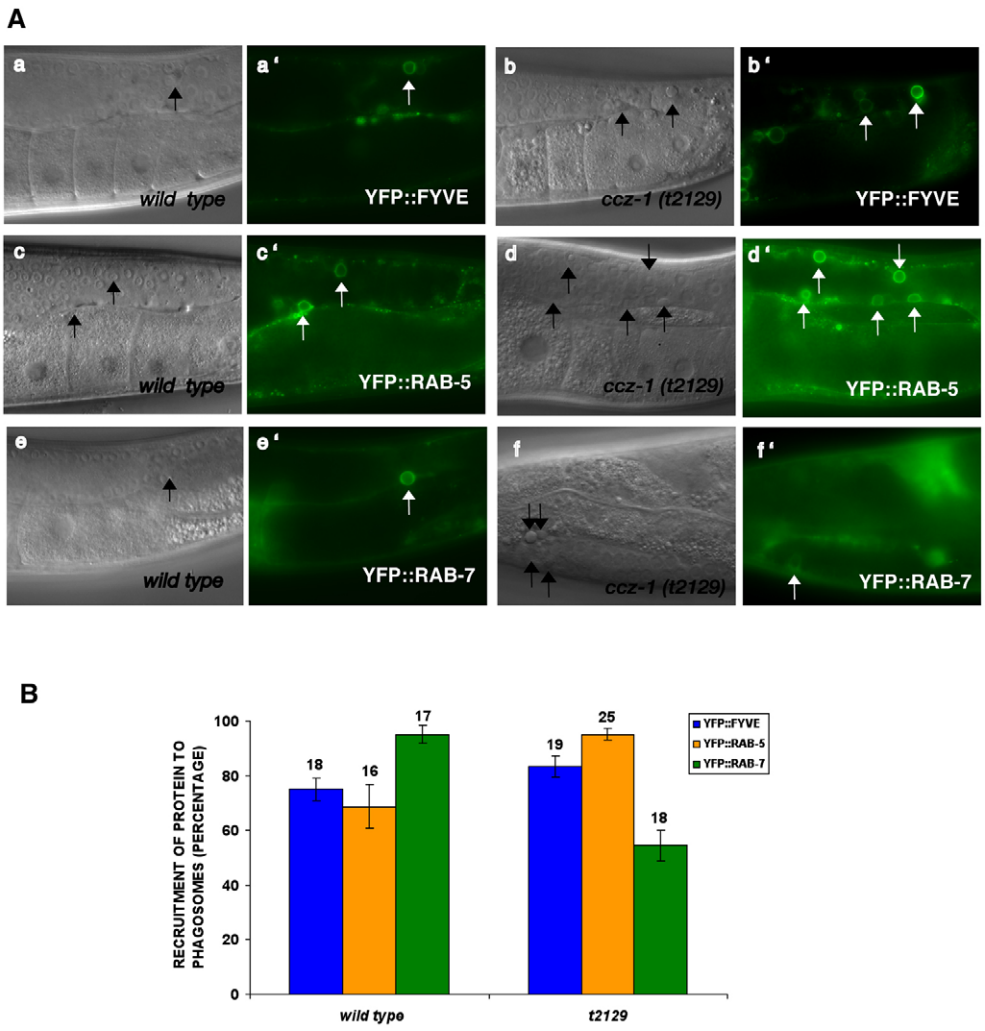

Fig. 4. Protein localization of phagosome-maturation components in *ccz-1* mutants. (**A**) In the wild type (a,a′), the YFP–2×FYVE construct is derived from EEA-1 and is bound to membranes containing PtdIns(3)*P* produced by VPS-34. This localization is similar in *ccz-1(t2129)* worms (b,b′). RAB-5–YFP is recruited normally in *ccz-1(t2129)* (d,d′) as compared with the wild-type worms. The recruitment of RAB-7–YFP is dramatically decreased in *ccz-1(t2129)* mutants (f,f′) compared with the wild type (e,e′). Arrows indicate corpses positive for YFP halos. (**B**) Quantified data showing the recruitment of YFP-FYVE, YFP–RAB-5 and YFP–RAB-7 around the germline corpses in a wild-type and in a *ccz-1(t2129)* background. Apoptotic corpses were counted under DIC optics and scored for YFP-positive halos under ultraviolet light. The result was expressed in percentage. Numbers over the columns refer to the number of analyzed worms in each case. Columns indicate means ± s.e.m.

fed with bacterial clones expressing dsRNA of different genes involved in the digestion pathway. Indeed, inactivation by RNAi of *dyn-1*, *vps-34* or *rab-5*, all of which function upstream of *rab-7*, completely abolished the recruitment of CCZ-1–YFP around the corpses (Fig. 3Bb-Bd′,C), indicating that the presence of these elements is necessary to load CCZ-1 onto the phagosome. However, inactivation of *rab-7* by RNAi allowed the recruitment of CCZ-1–YFP onto the apoptotic-corpse-containing phagosomes (Fig. 3Bf,Bf′,C). Furthermore, RNAi of members of the HOPS complex led to a recruitment of CCZ-1–YFP around the apoptotic-corpse-containing phagosomes, although this recruitment was partially impaired (Fig. 3Bg-Bi′,C). This result strongly indicates that CCZ-1 acts downstream of RAB-5 but upstream of RAB-7 in the digestion pathway. *rab-5* and *rab-7* were efficiently knocked down by RNAi, as indicated by the strong degree of accumulation of persistent undigested corpses in the gonad (supplementary material Table S1). The result is also consistent with previous reports that showed that the HOPS complex acts genetically downstream of RAB-7 (Kinchen et al., 2008; Yu et al., 2008), but in addition suggests that it partially contributes to the recruitment or stabilization of CCZ-1 on the phagosomes. The HOPS complex could stabilize the binding of CCZ-1 or alternatively could act in one of the several branches for CCZ-1 recruitment onto the phagosome.

The above results are based on the analysis of the location of the proteins on the maturing phagosomes. We next performed a functional assay. It has been described that a gain-of-function phenotype of the GTPase CED-10 (Rac) can be generated by overexpression of the *ced-10* gene. This GTPase overexpression does bypass the functional requirement for genes upstream in the cell-corpse phagocytosis pathway (Reddien and Horvitz, 2000; Cabello et al., 2010). We likewise analyzed the effect of overexpressing the GTPase RAB-7 by expressing its gene under the control of the *ced-1* promoter (which drives the expression of the transgene to the engulfing cells) in a *ccz-1(t2129)* mutant background and indeed found a partial but significant reduction in the number of germline-persistent corpses (supplementary material Table S1). This result support the finding that *rab-7* acts genetically downstream of *ccz-1*.

It has been shown that *vps-18* (a member of the HOPS complex) is involved in endosome and lysosome biogenesis, both of which are processes affecting the digestion of apoptotic corpses (Xiao et al., 2009). Our results suggest that, similar to *vps-18* and *rab-7*, but different to *sand-1* (Poteryaev et al., 2007), *ccz-1* mediates phagosome maturation and lysosome formation. The accumulation of the yolk proteins fused to GFP (VIT-2–GFP) in large and peripheral granules in *ccz-1(t2129)* mutant oocytes and embryos indicates that the early steps of receptor-mediated endocytosis were normal. Thus, the disruption of *ccz-1* in *C. elegans* leads to late defects in the endocytic pathway after normal internalization of membranes and solutes. The pathway involved in lysosome biogenesis seems to be also affected in *ccz-1(t2129)* mutant worms, as indicated by the following two observations. First, the autofluorescent gut granules were abnormal in *ccz-1(t2129)* embryos where the maternal and the zygotic product of the gene was disrupted and, second, AO staining of the intestine to mark the acidic compartments revealed that the lysosomes were mostly absent in the *ccz-1(t2129)* mutants (supplementary material Fig. S5).

Our results support a model in which *C. elegans* CCZ-1, the homologue of *S. cerevisiae* Ccz1p, plays a role in lysosome biogenesis and phagosome maturation during digestion of apoptotic corpses, a hitherto unknown feature of this protein. Both functions are closely associated and the strong blockage of the degradation of phagosomal corpses, in the absence of CCZ-1, could be ultimately caused by the absence of functional lysosomes. CCZ-1 acts genetically downstream of the GTPase RAB-5 and upstream of the GTPase RAB-7, mediating the exchange of these GTPases during phagosome maturation. CCZ-1 requires RAB-5 to be recruited onto the phagosomes and is required for proper RAB-7 localization on the phagosomes (Fig. 3A). CCZ-1 also mediates vesicle transport in *C. elegans*, as shown by the other defects observed in the mutants. In the absence of CCZ-1, the endosomes do not fuse to the few lysosomes present in the cell and the yolk is not metabolized. Embryos then die ‘by starvation’. In addition, similar to Wnt-pathway mutants, *ccz-1* mutant embryos show defects in spindle orientation (Rocheleau et al., 1997; Thorpe et al., 1997). These defects seem to be independent of lysosome biogenesis because they are also observed in *sand-1* mutants. Wnt signalling induces the asymmetric localization of proteins such as Frizzled or β-catenin in receptor cells. This polarity, underlying spindle orientation and fate diversification (Goldstein, 2000; Park et al., 2004; Nakamura et al., 2005), depends on localized endocytosis, transport and recycling mediated by the retromer complex (Coudreuse and Korswagen, 2007; Hardin and King, 2008; Seaman, 2005). Such a process could become compromised in the absence of CCZ-1. Indeed, *dyn-1* mutants (the most upstream element in the corpse digestion pathway) contain arrested phagosomes without recycling of the phagocytic receptor CED-1 back to the plasma membrane (Kinchen et al., 2008).

These findings define novel requirements for CCZ-1, a widely conserved protein that mediates key processes in the normal development of an organism, such as yolk metabolism, cell-cell signalling or digestion of the apoptotic corpses. Studying cell-corpse degradation is important not only for understanding a general biological process in animal development but also because efficient clearance of apoptotic cells protects humans from several diseases.

Materials and Methods

Strains and genetics

Animals were grown at 25°C as described (Brenner, 1974). The following mutations were used: LG II, *rab-7(ok511)*; LG IV, *sand-1(ok1963)*; LG V, *ccz-1(t2129)*, *ccz-1(t2070)*, *ccz-1(t2170)*, *ccz-1(ok2182)*, *dpy-11(e224)*. Other genes used in this work were inactivated using RNAi. Genetic mapping of mutations was carried out as described (Davis and Hammarlund, 2006).

RNAi

dsRNA-mediated gene interference was performed by feeding (Fraser et al., 2000). Synchronized L1 larvae were spotted onto feeding plates and incubated at 15°C until the gonads of adults and embryos of the next generation could be scored.

Light microscopy

Apoptotic corpses were detected by their refractile shape under a DIC light microscope and scored as described (Schnabel et al., 1997; Reddien and Horvitz, 2000). Fluorescent microscopy of transgenic worms was performed using a Zeiss Axioplan 2 microscope equipped with DIC optics and standard epifluorescence. Staining of worms with AO (Molecular Probes) was performed as described previously (Kinchen et al., 2005).

For the 4D microscope analysis of embryos, 25 focal planes of the embryos were recorded over 10 hours. As a result, 4D movies (3D of the embryo + time) were obtained for each genotype. The SIMI Biocell software (SIMI GmbH, Germany) allowed tracing each and every cell of the embryo in time and space, as well as tracing of the mitosis and the terminal differentiation of each cell.

Electron microscopy

C. elegans grown on *Escherichia coli* plates were high-pressure frozen with an EM Pact2 device (Leica Microsystems, Vienna, Austria) as described by the manufacturer. The frozen specimens were freeze-substituted in anhydrous acetone containing 2% OsO_4 in a Leica EM AFS2 freeze-substitution unit (Leica Microsystems). Specimens

were successively kept at –90°C, –60°C, and –30°C for 8 hours each, finally reaching room temperature. OsO_4 was removed by washing the specimens with anhydrous acetone twice. Subsequently, the specimens were gradually embedded in Epon/Araldite (Sigma-Aldrich, Buchs, Switzerland) prior to polymerization at 60°C for 48 hours. Thin sections were stained with aqueous uranyl acetate 2% and Reynolds lead citrate and imaged with a Phillips CM 100 transmission electron microscope (FEI, Eindhoven, Netherlands) using a Gatan Orius CCD camera and digital micrograph acquisition software (Gatan GmbH, Munich, Germany).

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