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遺傳分析 *C01F4.2/Arhgap6* 基因於線蟲細胞吞噬作用中所扮演之角色

Genetic analysis of the role of *C. elegans* *C01F4.2*/*Arhgap6* in engulfment process

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本論文係學生 [redacted] 在國立臺灣大學生命科學院分子與細胞生物學研究所就讀期間，所完成之碩士論文，於民國 98 年 7 月 10 日經考試委員審查合格。

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中文摘要

清除凋亡細胞於計畫性細胞凋亡中是重要過程。在線蟲的研究中發現，於凋亡細胞吞噬過程中，有兩條訊息傳導路徑可活化 CED-10/Rac GTPase，其一為 *ced-1*, *ced-6*, *ced-7*; 另一為 *ced-2*, *ced-5*, *ced-12*，造成細胞骨架之重組以吞噬凋亡細胞。於哺乳類細胞株的研究中發現另外兩個同屬 Rho-family GTPase 的蛋白可調控細胞吞噬作用：RhoA 與 Cdc42。近年研究認為，特定 Rho-family GTPase 之活化與去活化在凋亡細胞吞噬過程中均伴演重要角色。這些調控具有時間性與空間性，且需要完善控制。為了尋找參與凋亡細胞吞噬過程中新的 Rho-family GTPase 之負向調控分子，我們篩檢了十六個於線蟲中會轉錄具有 RhoGAP domain 蛋白之基因突變體。我們發現，於 *C01F4.2(ok1316)*線蟲中，胚胎時期之凋亡細胞屍體數較野生種高。將 C01F4.2a 蛋白進行序列比對結果發現其與人類之 ARHGAP6 蛋白具高度相似性，此蛋白具有特定調控 RhoA 之活性。藉由分析細胞屍體之殘留時間，我們推測於 *C01F4.2(ok1316)*突變株中所增加之細胞屍體數乃導因於細胞屍體清除機能的缺失。於進一步的遺傳分析中，我們發現 *C01F4.2* 基因的缺失並不會增強吞噬基因突變株之性狀。由此我們推測 *C01F4.2* 應與兩條訊息傳導路徑之吞噬基因共同作用以調控凋亡細胞之吞噬過程。

重要名詞：計畫性細胞凋亡、細胞吞噬作用、Rho-family GTPases、GAP。

Abstract

Clearance of apoptotic cells is an ultimate step of programmed cell death. In *C. elegans*, two partially redundant signaling pathways, *ced-1*, *ced-6*, *ced-7* in one, and *ced-2*, *ced-5*, *ced-12* in the other, converge at CED-10, a Rho-family small GTPase, leading to actin cytoskeleton rearrangement during the engulfment of apoptotic cells. Studies in mammalian cell lines also linked the function of two additional Rho-family GTPases (RhoA and Cdc42) to phagocytosis. Recent studies suggested that the activation and inactivation of specific Rho-family proteins is important during the engulfment of apoptotic cells. These regulations should be tightly controlled spatially and temporally. In order to identify novel negative regulator for Rho-family GTPases during engulfment process, we examined 16 potential *gap* (GTPase-activating protein) genes that encode protein with RhoGAP domain in *C. elegans* and identified *C01F4.2*. The *C01F4.2(ok1316)* mutant and *C01F4.2(RNAi)* have increased embryonic cell corpse number compared with that of wild-type. The C01F4.2a protein is similar to human ARHGAP6 protein, which has a GTPase-activating activity specific for RhoA. The cell corpse duration analysis showed that the elevated cell corpses number in *C01F4.2(ok1316)* embryos was due to the defect in the clearance of apoptotic cells. Our double mutant analysis showed that the *C01F4.2(ok1316)* mutation did not enhance the cell-corpse number of mutants defective in either of the two previously identified

engulfment pathways. Therefore *C01F4.2* appears to be required functions in both pathways to regulate cell corpses engulfment.

Keywords: Programmed cell death, Engulfment, Rho-family GTPases, GAP

Table of Contents

1. Introduction

Programmed cell death (apoptosis) is an evolutionary conserved cellular self-destruction process in many organisms. It acts as a mechanism to eliminate aberrant, damaged or unnecessary cells. After cells undergo apoptosis, clearance of apoptotic cells is an important process to preventing organisms from autoimmune disorder (Jacobson, Weil et al. 1997).

During the development of a *Caenorhabditis elegans* hermaphrodite, 131 of 1030 somatic cells and overly 300 germ cells would undergo programmed cell death. The dying event can be easily observed by scoring cell corpses, button-like objects with distinctive refraction (Figure 1a), under Normaski optics in living animals (Sulston and Horvitz 1977). Furthermore, the patterns of cell lineage is almost invariant in different individuals (Sulston and Horvitz 1977; Sulston, Schierenberg et al. 1983), we could examine whether animals have defect in programmed cell death by scoring the number of cell corpses in embryonic developmental stages (Figure 1b). This becomes a great advantage for studying programmed cell death by using *C. elegans* as a model organism.

Previous studies have defined programmed cell death into four integrating phases: specification, execution, engulfment and degradation (Steller 1995).

During the specification phase, genes (*ces-1*, *ces-2*, *tra-1*, *hlh-2/3*) involved in determination of death cell fate express in specific cells destined to die, and finally

results in the induction of pro-apoptotic BH3-only gene *egl-1*(Egl, EGg-Laying defective) (Metzstein, Hengartner et al. 1996; Conradt and Horvitz 1999; Thellmann, Hatzold et al. 2003). Of the 131 cells that destined to die, most of them are of neuronal origin. While programmed cell death is blocked, most would survive and function as neuron cells (Ellis and Horvitz 1986). In addition, a small number of hypodermal cells, muscle cells and the sisters of the pharyngeal gland cells die as well (Conradt and Xue, 2005).

During the execution phase, expression of *egl-1* gene leads to accumulation of EGL-1 protein in cells destined to die (Conradt and Horvitz 1998; Bouillet and Strasser 2002). Binding of EGL-1 to CED-9 (Ced, CEll-Death abnormal), a protein orthologue of human proto-oncogene product Bcl-2, leads to conformational change and release of CED-4 (Conradt and Horvitz 1998; del Peso, Gonzalez et al. 1998; Parrish, Metters et al. 2000; Yan, Gu et al. 2004). CED-4 is a protein similar to human Apaf-1(apoptotic protease activating factors), an activator of human Caspase-9. Released CED-4 undergoes oligomeriazation and promote activation of CED-3, which execute cell death (Yang, Chang et al. 1998).

After apoptosis, dying cells would be engulfed and degraded for elimination of cytotoxic substrates and reuse of resourses. For the lacking of professional phagocytic cells in *C. elegans*, cells neighboring to the dying cells act as engulfing cells to engulf

them (Robertson and Thomson 1982; Sulston, Schierenberg et al. 1983). Hypodermal cells, pharyngeal muscle cells, gonadal sheath cells and intestine cells have been reported to be competent engulfing cells (Sulston and Horvitz 1977; Sulston, Schierenberg et al. 1983; Hoeppner, Hengartner et al. 2001). Genetic studies in *C. elegans* have discovered seven genes involved in engulfment phase of programmed cell death and can be separated into two redundant pathways. *ced-1*/*Srec*, *ced-6*/*Gulp*, *ced-7*/*Abc1* in one and *ced-2*/*CrkII*, *ced-5*/*Dock180*, *ced-12*/*Elmo-1*, and *ced-10*/*Rac1* in the other (Wu and Horvitz 1998; Wu and Horvitz 1998; Liu and Hengartner 1999; Reddien and Horvitz 2000; Gumienny, Brugnera et al. 2001; Lundquist, Reddien et al. 2001; Wu, Tsai et al. 2001; Zhou, Hartwieg et al. 2001). Activation of CED-10 in engulfing cells regulates actin cytoskeleton rearrangement, which in turn promotes extension of pseudopod to embrace dying cells.

During the degradation phase, the engulfed cells would be incorporated into an organelle called phagosome, which in turn fuses with lysosome to become phagolysosome, an organelle containing nucleases and enzymes involved in nuclear DNA degradation (Zhou and Yu 2008). In recent years, the large GTPase *dyn-1* (DYNamin-related) and the small GTPase *unc-108*/*Rab-2*(Unc, UNCoordinated), *rab-5* (RAB family) and *rab-7* are found to promote phagosome maturation by mediating recruitment and fusion of lysosome to phagosome (Yu, Odera et al. 2006; Kinchen,

Doukoumetzidis et al. 2008; Lu, Zhang et al. 2008; Mangahas, Yu et al. 2008). Null mutation in engulfment pathway component *ced-1* not only results in a delay of engulfment but degradation, which suggest a signaling pathway initiated by *ced-1* and regulates activation of downstream GTPases that involved in degradation (Yu, Lu et al. 2008). Taken together, a successful clearance of apoptotic cells requires normal function and coordination of engulfment genes and degradation genes.

In *C. elegans* engulfment process, actin cytoskeleton reorganization is crucial in promoting the extension of pseudopod to embrace the dying cell. Previous studies linked the Rho-family small GTPases to actin cytoskeleton rearrangement during many types of cellular movement (Ridley 2001). The best-studied Rho-family GTPases are RhoA, Rac, and Cdc42. These GTPases cycle between GTP-bound and GDP-bound states and become active or inactive, respectively. There are three types of regulatory proteins help the cycling of Rho GTPases: guanine nucleotide exchange factors (GEFs), which promotes the activation of GTPases by changing GTP for GDP; GTPase activation proteins (GAPs), which enhance the intrinsic GTP hydrolysis activity to inactivate Rho proteins; and guanine-nucleotide-disassociation inhibitors (GDIs), which bind to GDP-bound Rho proteins and therefore prevents the interaction with regulatory molecules (Buchsbaum 2007).

Recent studies suggested that the activation and inactivation of specific

Rho-family proteins is important during the engulfment of apoptotic cells. These regulations should be tightly controlled spatially and temporally. When engulfment happens, the activated Rac1 was recruited to form phagocytic cups that were comprised of actin patches. When the phagocytic cup was closed, Rac1 was down-regulated, and the actin patches were abruptly broken down. They also found that expression of constitutively activate Rac1 resulted in a significant delay of the closure of phagocytic cup (Nakaya, Kitano et al. 2008). A combination of studies in mammals and *C. elegans* revealed an evolutionary conserved pathway that leads to Rac1 (in *C. elegans*, CED-10) activation. This pathway involves an adaptor protein CrkII (in *C. elegans*, CED-2); a GEF for Rac1, Dock180 (in *C. elegans*, CED-5); and a cofactor ELMO1 (in *C. elegans*, CED-12). By monitoring the activity of Rac1 during engulfment, it has been found to be active during the extension of pseudopod. (Hoppe and Swanson 2004).

On the other hand, activation of RhoA seems to have a negative effect on engulfment process. Previous studies found that RhoA level decreases during phagocytosis. Furthermore, inhibition of RhoA enhances the uptake of apoptotic cells. Conversely, overexpression of constitutively active RhoA inhibits engulfment (Tosello-Trampont, Nakada-Tsukui et al. 2003).

During the engulfment process, active Cdc42 has been found only in the tip of the extending pseudopod (Hoppe and Swanson 2004). The Cdc42 effector WASP stimulates

the polymerization of actin when bound to GTP-Cdc42 (Rohatgi, Ho et al. 2000) and could regulate actin polymerization in the forming phagosome (Lorenzi, Brickell et al. 2000) However, the precise role and regulating mechanism of Cdc42 on engulfment is not clear at present.

In spite of numerous positive regulators of Rho-family GTPase have been identified, molecules that negatively regulating Rho GTPases still remain to be illustrated. To identify novel negatively regulatory molecules that inactivate Rho-family GTPases during engulfment process, we began a screen for *gap* (GTPase activation protein) genes by using *C. elegans* as a model organism. We examined 16 potential *gap* genes for Rho-family small GTPases and identified *C01F4.2*. The number of cell corpses in *C01F4.2(ok1316)* animals were found to be increased at four embryonic stages. BLAST of C01F4.2a protein sequence against human proteome identified human ARHGAP6 protein, which has a GTPase-activating protein activity specific for RhoA. By a time-lapse recording analysis, we found that the cell corpses persisted longer in *C01F4.2(ok1316)* animals than in wild-type, which suggested that the increased cell corpses was due to the defective clearance of apoptotic cells. In further genetic analysis, our results indicated that *C01F4.2* does not specifically act within either engulfment pathway. *C01F4.2* seems to function together with engulfment genes in both pathways to promote engulfment process in *C. elegans*.

2. Materials and Methods

2.1 *C.elegans* strains used and genetics

The Bristol strain N2 was used as wild type strain. The following mutant strains were provided by *C. elegans* Genetics Center (CGC, University of Minnesota, Minneapolis, MN) and listed by linkage groups: LG I: *C01F4.2(ok1316)*, *C16C2.4&ocrl-1(gk752)*; *ced-1(e1735)*; LG II: *rga-1(ok204)*, *syd-1(ju82)*; LGIII: *T04c9.1a(ok1510)*, *tag325(ok1330)*; LG IV: *ced-2(e1752)*, *ced-5(n1812)*, *ced-10(n3246)*, *ced-10(n1993)*; LG V: *rga-3(ok1889)*, *rga-4(ok1935)*; LG X: *rrc-1(ok1417)*, *rbg-1(ok1660)*. The other mutant strains are provided by Dr. Shoehi Mitani (Tokyo women's Colledge, Tokyo, Japan and listed by linkage groups: LG I: *hum-7(tm1882)*, *rlbp-1(tm3665)*; LGII: *gei-1(tm3626)*, *rbg-3(tm1910)*.

2.2 Maintaining *C. elegans*

All strains are maintained according to standard method (Brenner 1974). Briefly, worms grew at 20℃ on NGM (Nematode Growth Medium) plates. *E. coli* strain OP50 as food resource.

2.3 Quantification of cell corpses

C. elegans embryos were mounted on 4% agar with 40mM NaN_3. Embryonic cell corpses were scored by Normaski optics. Number of cell corpses at five developmental stages (comma, 1.5-fold, 2-fold, 3-fold, 4-fold) were assayed.

2.4 RNAi experiments

Induction of RNAi by feeding bacteria was performed as standard procedure. Bacteria containing RNAi constructs grew on NGM plates with 1mM IPTG and ampicillin to induce double-stranded RNA. L4 worms were transferred to RNAi plates and grew for 48 hours at 20℃.

2.5 4D analysis of corpse duration time

C. elegans embryos were mounted on 4% agar with M9 buffer solution. Embryos at about 200-cells developmental stage were scored until developing into 2-fold stage. Room temperature were maintained at 20℃ during time lapse recording. 41~45 DIC images with different focal planes were captured every minutes under Zeiss Axioskop2 mot plus microscopy and Axiovision Rel. 4.6 softwere. Duration of cell corpses were determined by time interval between appearance and disappearance of distinctive cell corpse morphology.

3. Results

3.1 *C01F4.2(ok1316)* mutant has increased embryonic cell corpse number

In *C. elegans*, *ced-10*, *rho-1* and *cdc42* encodes protein orthologues to mammalian Rac1, RhoA and Cdc42 according to Wormbase information, respectively. These gene products belong to Rho family GTPases. Mutation on *ced-10* gene results in a deficiency of cell corpse engulfment in *C. elegans* (Lundquist, Reddien et al. 2001), while the defect of mutation on *rho-1* or *cdc42* on engulfment remains unclear. In order to identify potential *gap* genes that regulate these Rho-family GTPases, we searched for RhoGAP domain containing proteins in *C. elegans* by using SMART and swiss-prot database and identified 16 *gap* genes.

We first examined the number of embryonic cell corpses in 16 *gap* mutants we found. We found that all *gap* mutants, except for *C01F4.2(ok1316)*, have no significant difference on cell-corpses number when compared to that in wild-type. In *C01F4.2(ok1316)* mutant animals, the number of embryonic cell-corpses were increased at early developmental stages (Table 1). *C01F4.2(ok1316)* animal carries a 863 base-pair deletion from exon 9 to exon 11 according to the Wormbase information (Figure 2a). We designed primers to confirm the deletion in *C01F4.2(ok1316)* by PCR.

To further investigate how *C01F4.2* affects programmed cell death, we outcrossed *C01F4.2(ok1316)* three times and scored embryonic cell-corpse number from comma

stage to 4-fold stage. When compared to wild-type animal, the number of embryonic cell corpses were significantly increased in *C01F4.2(ok1316)* mutant at comma, 1.5-fold, 2-fold and 3-fold stages. However, no significant difference was observed at 4-fold stage between *C01F4.2(ok1316)* and that in wild-type (Table 2). We also did RNA interference (RNAi) on *C01F4.2* gene and obtained a similar result. These results suggest that function of *C01F4.2* gene is required to early embryonic stages.

3.2 *C01F4.2* encodes a protein similar to human ARHGAP6

C. elegans C01F4.2 encodes a longer protein isoform C01F4.2a and a short isoform C01F4.2b. Predicted result from SMART database showed that C01F4.2a possesses a putative RhoGAP domain from residue 529 to 686, and there were no other apparent domains or motifs at N-terminal or C-terminal region (Figure 2b). Our previous study showed that *C01F4.2(ok1316)* animal has increased embryonic cell corpse number. The deleted region of *C01F4.2(ok1316)* allele started from residue 389 and ended at 613, which has a partial overlap with predicted RhoGAP domain (Figure 2c). Therefore, translated C01F4.2a in *ok1316* animal might be loss-of-function protein due to loss of GTPase-activating activity or protein misfolding.

BLAST of C01F4.2a protein sequence against human proteome identified human ARHGAP6 protein, which has a GTPase-activating protein activity specific for RhoA. It has been reported that ARHGAP6 can function as a cytoskeletal protein that promotes

actin remodeling and might be involved in engulfment process (Prakash, Paylor et al. 2000). We then did a sequence alignment of RhoGAP domain between *C. elegans* C01F4.2a and longer isoform of human ARHGAP6 (ARHGAP6 isoform 1). Data showed that RhoGAP domain of these two proteins are highly conserved (with 43% identity and 74% similarity) (Figure 3). Furthermore, the catalytic residue of RhoGAP domain (R551) was also conserved, suggesting that the C01F4.2a might also have a GAP activity.

3.3 The elevated cell corpse number in *C01F4.2(ok1316)* might be caused by failure of corpse removal

The increase of embryonic cell corpses in *C01F4.2(ok1316)* could be caused by excessive cell death or defects in cell-corpse clearance. For the difficulties on tracing dying events in embryonic developmental stages. We first examined whether *C01F4.2(ok1316)* mutants have a defect in cell-corpses clearance.

A time-lapse recording of wild-type and *C01F4.2(ok1316)* embryos showed that the duration time of cell corpse is elongated in *C01F4.2(ok1316)* mutant compared with wild-type (Figure 4). In wild-type embryos, cell corpses were removed within half an hour in average (26±12 minutes). However, the time interval between appearance and disappearance of distinct cell corpse morphology was elongated to 43±23 minutes in average in *C01F4.2(ok1316)* embryos. Moreover, no cell corpse in wild-type embryos

lasted for more than 60 minutes, while in *C01F4.2(ok1316)* mutant we observed persistent cell corpse for more than 100 minutes.

This result suggested that the elevated cell-corpses number in *C01F4.2(ok1316)* might be due to failure of corpse removal. However, we could not rule out the possibility of excessive cell death events in *C01F4.2(ok1316)* compared to wild-type animal.

3.4 *C01F4.2* functions together with engulfment genes to promote cell corpse clearance

Since the elevated cell corpses number in *C01F4.2(ok1316)* was due to defective cell corpses clearance, we wondered if *C01F4.2(ok1316)* functions together with engulfment genes to promote cell corpse engulfment. In *C. elegans*, there are two redundant pathways which regulating engulfment process: *ced-1*, *ced-6*, *ced-7* in one and *ced-2*, *ced-5*, *ced-12*, *ced-10* in the other (Wu and Horvitz 1998; Wu and Horvitz 1998; Liu and Hengartner 1999; Reddien and Horvitz 2000; Gumienny, Brugnera et al. 2001; Lundquist, Reddien et al. 2001; Wu, Tsai et al. 2001; Zhou, Hartwieg et al. 2001). To test this possibility, we generated double mutants of *C01F4.2(ok1316)* with loss-of-function mutants in each pathway and scored the number of persistent cell corpses at five embryonic stages.

We first examined whether *C01F4.2(ok1316)* functions together with engulfment genes in the first pathway. When doubled to the first pathway mutants, *C01F4.2(ok1316)*

did not enhance the mutant phenotype in *ced-6(n1813)* and *ced-7(n1892)* background (Table 3). For the difficulty of generating *ced-1(e1735); C01F4.2(ok1316)* double mutant, we treated *C01F4.2* RNAi to *ced-1(e1735)* animals and scored the persistent cell corpse number at embryonic stages. Surprisingly, *C01F4.2* RNAi suppressed the phenotype of *ced-1(e1735)* mutant at 1.5-fold and 2-fold stages while other stages remained unchanged.

We then examined if *C01F4.2* functions together with engulfment genes in the second pathway. When doubled to the gene members in the second pathway, *C01F4.2(ok1316)* did not significantly enhance the mutant phenotype in *ced-2(e1752)* and *ced-5(n1812)* background (Table 3).

Our results indicated that *C01F4.2(ok1316)* did not act specifically within either pathway. These findings suggested that *C01F4.2* functions together with engulfment genes in both pathways and regulating the clearance of cell corpses. Since the loss of C01F4.2 function did not enhance the engulfment defect in loss-of-function mutants of engulfment genes in both pathways, we suggested that C01F4.2 might not involved in the initiation state of cell-corpses engulfment and plays a minor role during engulfment of apoptotic cells.

4. Discussion

We have investigated the role of *C. elegans C01F4.2* in regulating the engulfment process during programmed cell death. We found that *C01F4.2* positively regulates the engulfment process. Our double mutant analysis suggested that *C01F4.2* functions together with the two known pathways during engulfment of apoptotic cells.

4.1 How do *C01F4.2* work during engulfment process?

Cytoskeleton rearrangement is a crucial event in several cellular processes including changing of cell shape, cell migration and engulfment. Several small GTPase family members have been found to be participated in these processes. (Ridley 2001).

In a screening conducted by Nakaya et al., RhoG, Rab5, and Rac1 were found to enhance the engulfment of apoptotic cells. Conversely, RhoA inhibited the process (Nakaya, Tanaka et al. 2006). However, in the cell movement of neutrophils, both Rac1 and RhoA are activated at the front and rear of the cells respectively (Xu, Wang et al. 2003). These findings suggest that the specific regulation of small GTPases is important in promoting cytoskeleton rearrangement, which should be function at the right place and the right time. In cells, a regulating network which consists of GEFs, GAPs, and GDIs acts coordinately to activate and inactivate these small GTPases. Properly interactions between these regulatory molecules are important to regulate their targets spatiotemporally.

According to the reviewing paper by Ravichandran and Lorenz, reciprocal activation of Rac1 and RhoA happens during engulfment of apoptotic cells (Ravichandran and Lorenz 2007). There is a signaling complex consists of CrkII, Dock180, and ELMO1, which leads to the increase of GTP-bounded Rac1. Meanwhile, the GTP-bounded RhoA decreases in parallel, which probably in turn decreases the signaling about stress-fibre formation to facilitate cell shape change during pseudopod extension. This inactivation might be regulated by GAP proteins specifically for RhoA. In a study by Prakash et al., human ARHGAP6 was found to be having GAP activity for RhoA but not Rac and Cdc42. They also suggested ARHGAP6 can function as a cytoskeletal protein to promote actin cytoskeleton remodeling, which might be involved in the engulfment process (Prakash, Paylor et al. 2000).

In this study, we found that *C01F4.2* encodes a protein similar to the longer isoform of human ARHGAP6; *C01F4.2* was also found to be involved in the *C. elegans* engulfment process, which suggested a evolutionary conserved role of *C01F4.2/Arhgap6* in regulating *RhoA* (in *C. elegans*, *rho-1*) during engulfment of apoptotic cells. Surprisingly, the number of cell corpses in *rho-1(RNAi)* animals was found to be increased when compared to that in wild-type during embryogenesis (This phenotype was observed by another lab member, Hsiao-Han Hsieh), suggesting that *C.elegans* RHO-1 and mammalian RhoA might play different roles during engulfment of

apoptotic cells. The actual function of RHO-1 in engulfment and the relationship between C01F4.2 and RHO-1 in *C. elegans* still remains to be illustrated. Furthermore, how does *C01F4.2* affect the actin cytoskeleton rearrangement should be further analyzed by monitoring actin cytoskeleton marker. For example: rhodamine phalloidin or fluorescent protein fusioned actin.

4.2 What's the relationship between *ced-1* and *C01F4.2*?

In *C. elegans*, CED-1, CED-6, and CED-7 act within the same genetic pathway during engulfment of apoptotic cells (Mangahas and Zhou 2005). In our double mutant analysis between *C01F4.2* and *ced* mutants defective in engulfment, we observed an unexpected decrease of the number of cell corpses in *ced-1(e1735); C01F4.2(RNAi)* at 1.5-fold and 2-fold stages. However, *C01F4.2(ok1316)* mutation did not modify the engulfment defect of *ced-6(n1813)* and *ced-7(n1892)* mutants (Table 3). In recent years, *C.elegans* Abl-1 was found to inhibit cell engulfment (Hurwitz, Vanderzalm et al. 2009). By quantification of cell-corpses number, they found that the *abl-1* mutations suppressed the engulfment defect of engulfment *ced* gene mutants in both pathways, which including *ced-1(e1735)*. They propose that ABL-1 inhibit the engulfment via a pathway that distinct from the two known engulfment pathways. Since *C01F4.2* was found to act together with engulfment genes in two known pathways in our study, the suppression on *ced-1(e1735)* defect between *ced-1(e1735); abl-1(n1963)* and *ced-1(e1735);*

C01F4.2(RNAi) might be caused by distinct mechanism.

In our study, the observation that loss of *C01F4.2* leads to different consequences on these mutants might be caused by different effects between RNAi and mutation of *C01F4.2* gene. The *C01F4.2(ok1316)* mutant might express partial loss-of-function C01F4.2, which still possesses few activities of normal protein. In *ced-1(e1735); C01F4.2(RNAi)*, the expression of C01F4.2 were fully abolished by RNA interference, therefore results in a different phenotype when compared to *C01F4.2ok1316); ced-6(n1813)* and *C01F4.2(ok1316); ced-7(n1892)*.

The suppression of *ced-1(e1735)* phenotype might be caused by a rescuing effect induced by active RHO-1. In *ced-1(e1735); C01F4.2(RNAi)* animals, the loss of C01F4.2 might leads to the persistent activation of RHO-1, which transduce a signal to a pathway downstream of *ced-1* and bypass the defect caused by loss of CED-1 function. In *C. elegans*, DYN-1 has been reported to mediate signal of CED-1 and promote the delivery and incorporation of intracellular vesicles to pseudopod, which provide materials for pseudopod extension (Yu, Odera et al. 2006). C01F4.2 and RHO-1 might also be involved in the regulating of DYN-1 mediated vesicular transportation to promote the engulfment process, not only by regulating actin cytoskeleton reorganization.

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6. Figures and Tables

Table 1.

The embryonic cell-corpse profile of mutants defective in genes coding for the GAP- domain containing protein.

Genotype	No. of cell corpses				
	comma	1.5 fold	2 fold	3 fold	4 fold
N2	10.6±2.0	8.9±2.3	8.6±1.3	1.8±1.3	0.3±0.5
rrc-1(ok1417)	10.7±2.0	9.8±2.0	9.0±2.0	2.6±1.1	1.1±0.9*
syd-1(ju82)	12.1±3.2	14.3±3.5*	9.4±1.3	3.8±1.9*	0.5±0.6
rbg-1(ok1660)	11.3±3.3	9.5±1.3	8.3±2.5	3.8±1.5*	0.8±0.7
rbg-3(tm1910)	10.0±2.2	9.8±1.9	9.0±2.8	2.6±0.5	0.3±0.5
rga-1(ok204)	11.0±3.0	10.7±1.9	11.0±3.3	2.3±1.3	0.4±0.5
rga-1(ok1630)	12.3±3.1	10.4±2.7	9.2±1.5	2.0±0.0	0.5±0.7
rga-2(hd102)	11.9±3.2	12.5±3.3*	10.0±1.8	1.3±0.7	0.4±0.7
rga-3(ok1889)	8.7±2.0	8.0±1.7	6.7±1.5	2.6±1.0	0.3±0.7
hum-7(tm1882)	13.4±2.4*	11.7±3.6	11.2±4.4	3.2±2.1	0.7±0.8
C01F4.2(ok1316)	14.7±3.4*	15.4±3.5**	15.9±3.1**	4.1±2.4*	0.3±0.5
ocrl-1(gk752)	11.4±2.7	12.6±2.5*	12.8±4.3	3.8±1.8	0.5±0.5
tag-325(ok1330)	10.4±1.9	9.4±2.1	9.3±1.2	0.7±0.5	0.5±0.7
T04c9.1a(ok1510)	10.0±3.7	10.3±1.7	10.3±5.1	2.4±2.1	0.4±0.5
rlbp-1(tm3665)	9.0±3.2	7.8±1.0	ND	ND	0.5±0.5
B0393.2(tm1910)	10.0±2.2	9.8±1.9	9.0±2.8	2.6±0.5	0.3±0.5
gei-1(tm3626)	9.6±2.7	8.7±2.0	10.7±3.1	3.0±0.9*	0.5±0.5

The values are shown as mean ± standard deviation. ND stands for not determined.

Cell-corpse number in GAP mutants were compared with the number in N2 (wild-type) by student T-test.

*$p < 0.05$, **$p < 0.001$.

Cell corpses from the indicated animals were scored at comma, 1.5-fold, 2-fold, 3-fold, and 4-fold stage (15 embryos at each developmental stage).

Table 2.

The number of cell corpses increased in outcrossed *C01F4.2(ok1316)* and *C01F4.2 (RNAi)* embryos.

Genotype	No. of cell corpses				
	comma	1.5 fold	2 fold	3 fold	4 fold
N2	10.6±2.0	8.9±2.3	8.6±1.3	1.8±1.3	0.3±0.5
C01F4.2(ok1316)	15.8±4.0*	13.4±2.9*	14.9±3.8*	3.8±1.4*	0.6±0.8
C01F4.2(RNAi)	15.7±2.8*	12.0±1.2*	15.0±2.8*	5.3±1.5*	0.4±0.5

The values are shown as mean ± standard deviation.

Cell corpse number in *C01F4.2(ok1316)* and *C01F4.2(RNAi)* were compared with the number in N2 by student T-test.

*$p < 0.05$, **$p < 0.001$

Cell corpses from the indicated animals were scored at comma, 1.5-fold, 2-fold, 3-fold, and 4-fold stage (15 embryos in each developmental stage).

Table 3.

C01F4.2(ok1316) does not enhance the engulfment defects in the mutants of both pathways.

Genotype	No. of cell corpses				
	comma	1.5 fold	2 fold	3 fold	4 fold
C01F4.2(ok1316)	15.8±4.0	13.4±2.9	14.9±3.8	3.8±1.4	0.6±0.8
ced-1(e1735)	20.3±2.9	27.4±2.3	32.5±2.3	26.3±3.2	22.0±3.2
ced-1(e1735) C01F4.2(RNAi)	21.0±3.0	17.8±1.3**	27.0±2.3**	25.8±2.9	20.1±4.5
ced-6(n1813)	23.0±4.4	26.3±2.1	36.3±1.2	33.1±2.7	25.3±2.4
C01F4.2(ok1316); ced-6(n1813)	22.2±2.6	25.7±5.6	36.3±3.0	30.4±1.5*	24.2±1.8
ced-7(n1892)	24.4±3.0	29.6±4.3	34.3±4.2	31.8±3.4	27.8±4.0
C01F4.2(ok1316); ced-7(n1892)	23.7±1.9	29.0±2.4	35.0±2.6	31.2±2.9	28.0±3.3
ced-2(e1752)	16.0±3.6	17.4±1.8	26.8±4.1	21.0±3.2	22.2±3.6
C01F4.2(ok1316); ced-2(e1752)	19.0±3.4	19.4±2.1	29.2±3.5	25.6±1.9*	21.8±2.8
ced-5(n1812)	31.6±3.9	39.2±2.1	42.2±3.9	35.4±2.9	30.7±2.4
C01F4.2(ok1316); ced-5(n1812)	31.6±5.1	40.7±1.5	42.4±4.4	35.9±3.2	31.0±3.8

The values are shown as mean value ± standard deviation.

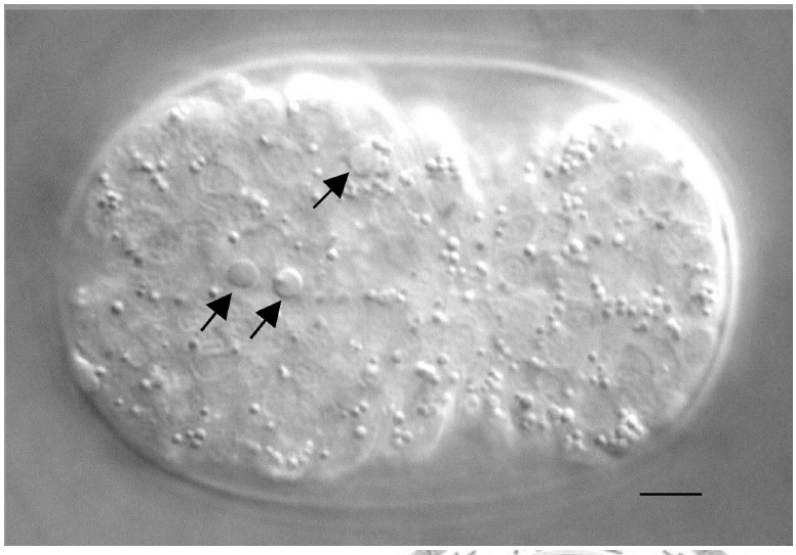
Cell corpse number in double mutants were compared with the number in *ced* single mutants by Student T-test.

*$p<0.05$, **$p<0.001$

Cell corpses from the indicated animals were scored at comma, 1.5-fold, 2-fold, 3-fold, and 4-fold stage (10 embryos in each developmental stage).

Figure 1.

(a) Morphology of cell corpse in an embryo of *C. elegans* under Normaski optics.



Arrows indicate three cells that underwent programmed cell death and have a raised-button-like morphology. The bar represent 5μm. This figure was adapted from Conradt, B. and Xue D. Programmed cell death (October 06, 2005), *WormBook*.

(b) Embryonic developmental stages of *C. elegans*.

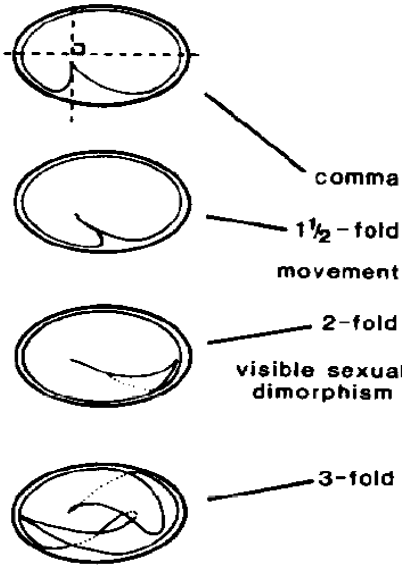
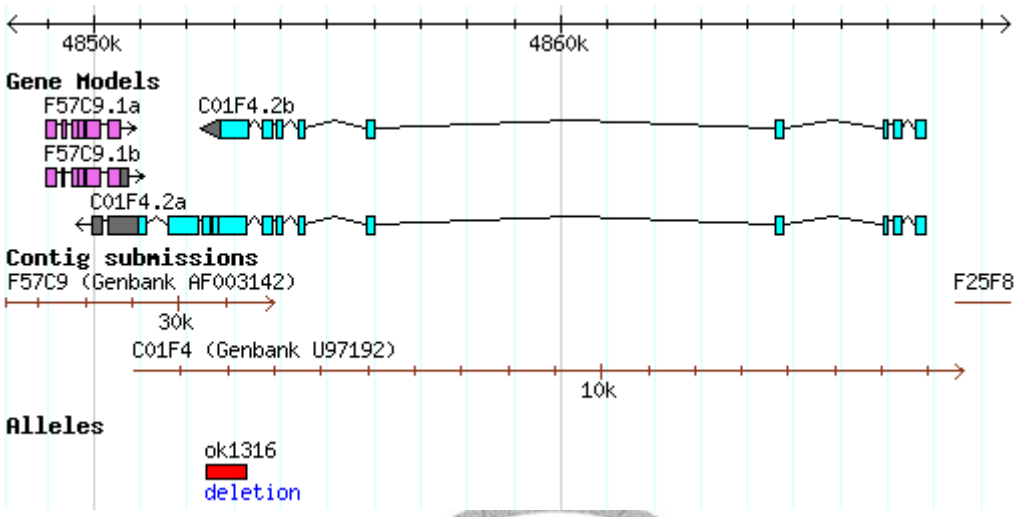


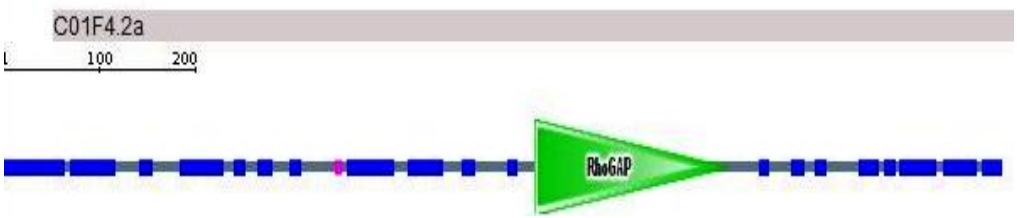
Figure 2.

(a) gene structure of *C014.2* in *C. elegans*



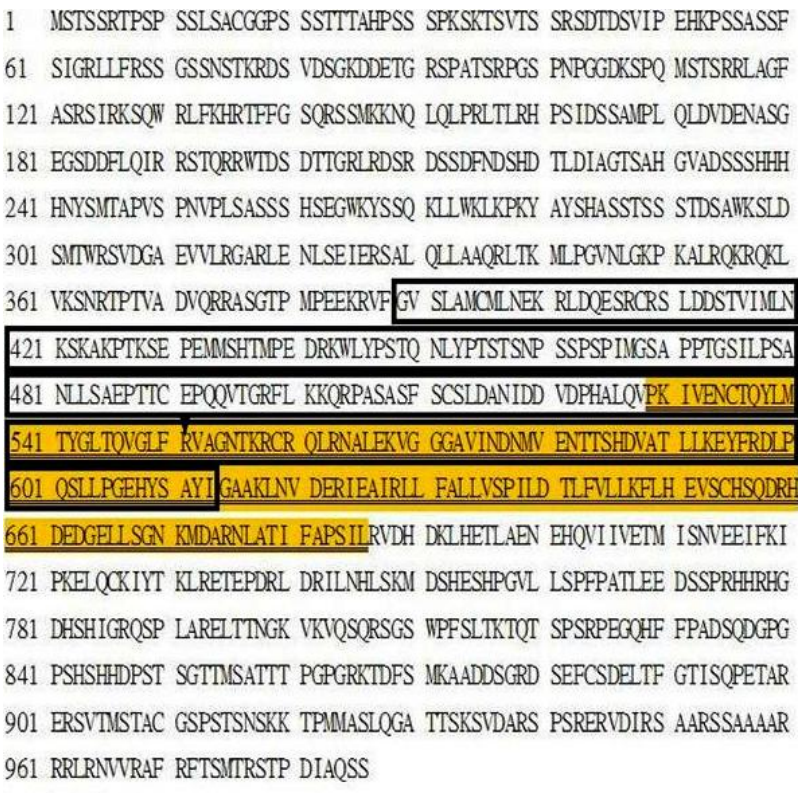
The blue boxes indicate the position of exons, black lines indicate the position of introns. The red box indicates the position of deleted region in *ok1316* allele, which spans exon 9 to exon 11 of *C01F4.2a*. This picture was adapted from Wormbase.

(b) Predicted protein structure of C01F4.2a by SMART database.



The green triangle indicates the position of RhoGAP domain in C01F4.2a.

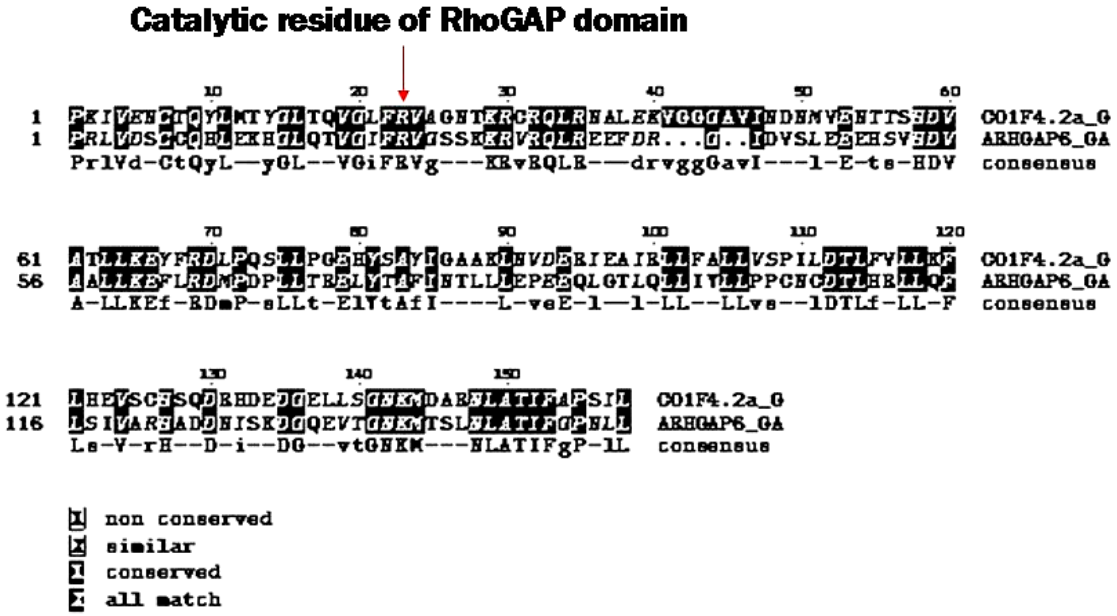
(c) Amino acid sequence of C01F4.2a protein in *C. elegans*.



Deleted region of *C01F4.2(ok1316)* allele (amino acid 389-613) is boxed. The putative RhoGAP domain is shaded by yellow box and the conservative catylytic residue (R551) of RhoGAP domain is indicated by arrow head.

Figure 3.

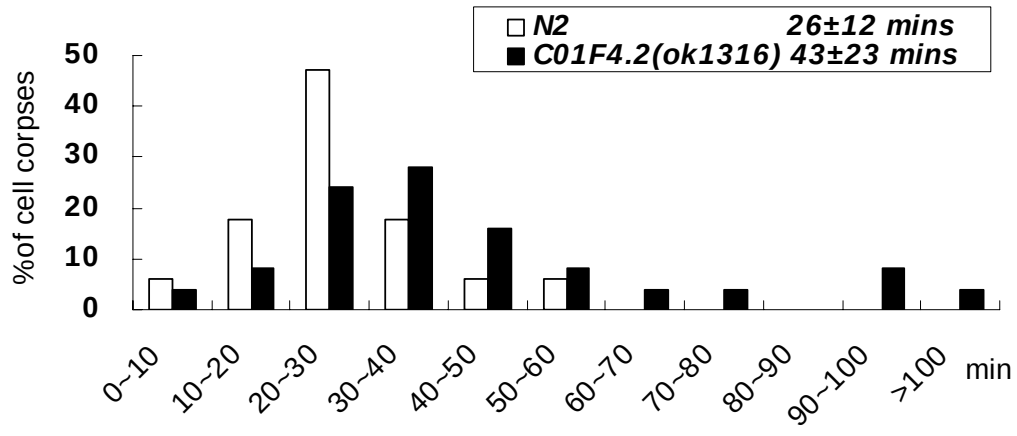
Alignment of *C. elegans* C01F4.2a GAP (GTPase activationg protein) domain with human ARHGAP6 isoform 1 GAP domain.



Amino acids shaded by black boxes indicated that identical residues exist in C01F4.2a GAP domain and human ARHGAP6 GAP domain.

Figure 4.

Four-dimensional microscopy analysis of durations of persistence of cell corpses.



The persistence of 30 cell corpses each from N2 embryos and *C01F4.2(ok1316)* embryos was analyzed (n=3). The numbers in parentheses indicate the average persistence for cell corpses ± standard deviation from each genotype. The y axis indicates the percentage of cell corpses within a specific duration range; the x axis indicates the cell-corpse duration time.