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Functional analysis of the single calmodulin gene in the nematode *Caenorhabditis elegans* by RNA interference and 4-D microscopy

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Calmodulin-like proteins – C. elegans – cell lineage – embryonic lethality – RNA interference

Calmodulin (CaM), a small calcium-binding protein, is the key mediator of numerous calcium-induced changes in cellular activity. Its ligands include enzymes, cytoskeletal proteins and ion channels, identified in large part by biochemical and cell biological approaches. Thus far it has been difficult to assess the function of CaM genetically, because of the maternal supply in *Drosophila* and the presence of at least three nonallelic genes in vertebrates. Here we use the unique possibility offered by the *C. elegans* model system to inactivate the single CaM gene (*cmd-1*) through RNA interference (RNAi). We show that the RNAi microinjection approach results in a severe embryonic lethal phenotype. Embryos show disturbed morphogenesis, aberrant cell migration patterns, a striking hyperproliferation of cells and multiple defects in apoptosis. Finally, we show that RNAi delivery by the feeding protocol does not allow the efficient silencing of the CaM gene obtained by microinjection. General differences between the two delivery methods are discussed.

Abbreviations. CaM Calmodulin – dsRNA Double-stranded RNA – NMY non-muscle myosin – RNAi RNA interference

Introduction

Calmodulin (CaM) is probably the best studied member of the EF hand family of eukaryotic calcium-binding proteins. CaM controls a large number of proteins and enzymes that share little sequence similarity in their binding sites and brings them under calcium control (for recent reviews see (Snedden and Fromm, 1998; Chin and Means, 2000; Hoeflich and Ikura, 2002)). Animals and plants display also several calmodulin-like (CaM-like) proteins, which are either rather closely (see for instance (Koller and Strehler, 1988)) or more distantly (see for instance (Haeseleer et al., 2000; Karabinos and Bhattacharya, 2000)) related to CaM.

Genetic studies in the unicellular organisms *Saccharomyces cerevisiae* (Davis et al., 1986), *Schizosaccharomyces pombe* (Takeda and Yamamoto, 1987), *Paramecium tetraurelia* (Hinrichsen et al., 1986) and *Aspergillus nidulans* (Rasmussen et al., 1990) documented that CaM is an essential protein. The same has also been found in the multicellular organism *Drosophila* where the CaM null individuals survived embryogenesis and died within 2 days of hatching as first instar larvae. The lack of embryonic lethality probably arose from the maternal CaM (Heiman et al., 1996). Thus, the function of CaM in the embryo is not known.

Here we have tried to determine the role of CaM during early development of the nematode *C. elegans* which offers not only a completely characterized metazoan genome (*C. elegans* Sequencing Consortium, 1998) but also an easy approach to functional analysis using reverse genetics based on RNA interference (RNAi) (Fire et al., 1998). Our RNAi experiments, based on microinjection, show embryonic lethality due to the silencing of the single CaM gene of *C. elegans*. 4-D microscopy (Schnabel et al., 1997) was used to analyse the CaM RNAi phenotype on the single cell level.

In contrast to RNAi delivery by microinjection the feeding protocol does not allow an efficient silencing of the CaM gene. Thus the high-throughput analysis performed by feeding on all *C. elegans* genes reports no embryonic lethality for CaM RNAi and indicates only a slower growth and reduced fertility (Kamath et al., 2003). While we observe similar results for

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delivery by feeding our microinjection results firmly establish that calmodulin is an essential gene of *C. elegans*. Some differences between the two delivery methods are discussed.

Materials and methods

Sequence analyses

The BLAST program with standard parameters (Altschul et al., 1990) was used to search the complete *C. elegans* protein database WORMPEP at the Sanger Centre (www.sanger.ac.uk). Percent identity calculations and search for protein sequence motifs in the *C. elegans* sequences were performed using the GCG software programs GAP and MOTIFS, respectively.

Nucleic acid methods

Amplification of full-length coding regions of *C. elegans cmd-1*, *cal-2*, *cal-3* and *cal-4* and the partial coding sequence of the *C. elegans cal-1* gene lacking 171 nucleotides at the 5′ side (*C. elegans* genome project at http://www.sanger.ac.uk) was performed using the cDNA library and the conditions previously described (Karabinos et al., 2001). The following primers were used for amplification: *cmd-1* (sequence name T21H3.3) 5′-ATGGCCGATCAACTGACCGAGGAG-3′ and 5′-TTACTTGGTTGTCATCATGGTGACGAAC-3′; *cal-1* (sequence name C13C12.1) 5′-ATGGCGATCCCATCAAATCTTATG-3′ and 5′-TTATTGATTAGACATCATCTTTACGAAC-3′; *cal-2* (sequence name C18E9.1) 5′-ATGAGCAACGATCCTCGCCGTGTG-3′ and 5′-TCACTTTATGATGTCGCAATCATTG-3′; *cal-3* (sequence name M02B7.6) 5′-ATGTCGATTATGCGTACCAGGACC-3′ and 5′-TTAGCTGAAAATTTTGACAAACTC-3′; *cal-4* (sequence name T07G12.1) 5′-ATGTATCGCCAGGGAACCAATCAATCG-3′ and 5′-TTATTTGGTAGGTGGATTAGTGGAG-3′. The resulting single PCR fragments were cloned into the blunt-TOPO pCR-2.1 plasmid vector (Invitrogen, San Diego, CA, USA) and the inserts were sequenced on both strands. The cloned *cmd-1* cDNA was used as template for in vitro RNA synthesis performed essentially as described (Karabinos et al., 2001). dsRNA was injected into the gonads of wild-type N2 or *rrf-3* (Simmer et al., 2002) hermaphrodites. No differences between the two strains were observed in our experiments. The progeny produced 0 to 45 hours after injection was scored for hatching, larval development, locomotion and fertility. Control injections used M9 medium (Sulston and Hodgkin, 1988).

For the RNAi feeding experiments the cDNA for *cmd-1* was ligated between the T7 promoters of the L4440 feeding vector (Fraser et al., 2000).

Four-dimensional microscopy

The four-dimensional microscopy system used for lineage analysis is as described (Schnabel et al., 1997), but now uses digital picture storage. Analysis of the 4-D records was carried out with the program SIMI°Biocell© developed by R. Schnabel and SIMI (D-85705 Unterschleissheim).

RNA interference mediated by feeding

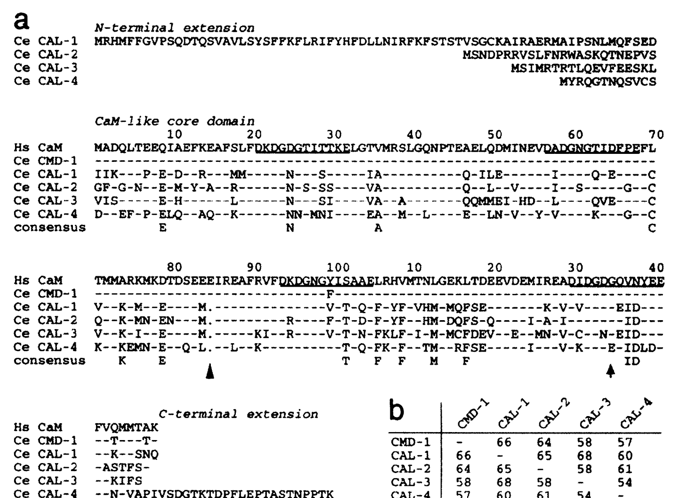
RNAi feeding experiments were performed according to Fraser et al. (2000) with the following modifications. The 5-cm NGM plates containing carbenicillin (25 μg/ml) and 1 mM IPTG were inoculated with 5 μl of the overnight *cmd-1*-cDNA/L4440-transformed HT115(DE3) bacterial culture and left for 6 hours at room temperature. The L3–L4 stage worms (F0) were washed with M9 medium and individually placed on the feeding plates and incubated for 48 hours at temperatures between 20–23 °C. The F0 worms were removed from the plates and their progeny (F1) was scored for abnormalities by Nomarski microscopy.

Results

Database search and the calmodulin family in *C. elegans*

A single calmodulin gene (*cmd-1*; T21H3.3) and four genes encoding CaM-like proteins were identified in the *C. elegans* WORMPEP database using the sequence of human calmodulin as a probe. Since CAL-1 (C13C12.1) was already identified (Salvato et al., 1986) we extended this nomenclature to CAL-2 (C18E9.1), CAL-3 (M02B7.6) and CAL-4 (T07G12.1). PCR amplification on a cDNA library from a mixed-stage population showed that all 5 genes are expressed. Sequence analysis of the cloned single PCR products provided the predicted mRNAs, except in the case of *cal-2*. The *cal-2* cDNA (GenBank accession number AJ512489) lacked an internal sequence of 50 nt starting with *gta* and ending with *cag*. Since the cDNA sequence predicts the conserved C-terminal end of CAL-2 (Fig. 1a) we conclude that the extra 50 nt in the database reflect a small intron previously not recognized by the *C. elegans* sequence consortium (1998).

CaM from man and *C. elegans* differ in only 2 residues located close to the C-terminus (Fig. 1a). All 4 CaM-like proteins have variable N-terminal extensions of 12 to 70 residues, which are absent from CaMs. CAL-4 has an additional C-terminal extension. These extensions have no significant similarity to any other protein in the NCBI non-redundant protein database. Over the central core regions CaM-like proteins differ in 50 to 61 residues from CaM but only 13 of these positions and a unique one residue deletion (Fig. 1a; arrowhead) are strictly conserved in all nematode CaM-like proteins. Within all 5 proteins, identity levels in the core domain range from 54 to 68% (Fig. 1b). Based on the program MOTIFS all proteins except for CAL-4 have 4 calcium-binding EF hands (Kawasaki



	CMD-1	CAL-1	CAL-2	CAL-3	CAL-4
CMD-1	-	66	64	58	57
CAL-1	66	-	65	68	60
CAL-2	64	65	-	58	61
CAL-3	58	68	58	-	54
CAL-4	57	60	61	54	-

Fig. 1. Alignment of human CaM (HsCaM) with *C. elegans* CaM (Ce CMD-1) and the four CaM-like proteins. (**a**) The three-domain structure of CaM-like proteins (CaM-like core and terminal extensions) is indicated. The locations of the Ca^{2+}-binding loops are *underlined*. Identical amino acids are depicted by *dashes*, deletions by a *dot*. The fourth EF-hand of CAL-4 may be non-functional in calcium binding due to the glutamate in the position marked by an *arrow*. The *arrowhead* and the consensus indicate the unique one-amino-acid gap and the 13 residues, respectively, shared only by the CaM-like proteins. (**b**) Pairwise comparison of sequence identities (%) of the core domains.

and Kretsinger, 1995). Due to the exchange of a conserved glycine by glutamate the last EF hand of CAL-4 seems inactive (Fig. 1a, arrow).

Calmodulin RNA interference through microinjection

The full-length coding sequence of *cmd-1* was used as template for in vitro dsRNA synthesis. The dsRNA was injected into the gonads of hermaphrodites (P0 generation) in order to induce specific dsRNA-mediated inhibition of gene expression (RNAi) (Fire et al., 1998). The next generation (F1), laid 0 to 45 hour after injection, was scored for defects in hatching, larval development, motility and fertility.

Injection of *cmd-1* dsRNA caused embryonic lethality with the highest penetrance of 95% observed in progeny produced between 28 and 45 h after injection (n = 177). The few animals that hatched either arrested as L1 larvae (2%) or grew very slowly with an uncoordinated phenotype (3%). This indicates an essential function for the single CaM gene *cmd-1* in nematode development, which cannot be substituted by the four CaM-like genes. In contrast to the embryonic lethality observed here in *C. elegans* by RNA interference, genetic experiments with *Drosophila* have shown that CaM null flies survive embryogenesis, but die within 2 days as first instar larvae (Heiman et al., 1996). While the lack of embryonic lethality in *Drosophila* is best explained through maternal CaM support (Heiman et al., 1996), we speculate that in our study a significant portion of the nematode maternal mRNA pool is depleted by RNA interference.

4-D microscopy of calmodulin RNAi embryos

To determine the role of CaM during nematode development in more detail, we analysed the phenotypes of 9 *cmd-1* RNAi embryos, which all arrested development at the premorphogenetic stage, with a 4-D microscope system (Schnabel et al., 1997). With this system first a 4D-recording (three dimensional time lapse) of an embryo is taken and then the records are replayed with the database SIMI°Biocell®, which allows the analysis of the development of embryos at the single cell level by documenting cell positions, cleavages and cell fates. The embryonic lineages of two embryos were reconstructed completely up to the premorphogenetic stage (Figs. 2 to 4, and supplementary information at http://www.tu-bs.de/institute/genetik/schnabel/refs.html). The lineages of the other 7 embryos were reconstructed in part in order to verify the observed defects. *cmd-1* RNAi embryos develop at a slightly slower rate (f = 1.3) than do normal animals. Defects in apoptosis, cell divisions, gastrulation, cell migrations and morphogenesis were scored.

cmd-1 RNAi terminal embryos displayed varying numbers of apoptotic cell corpses. The detailed lineage analyses showed that this is because the apoptotic programs are disturbed. Not only do some normal programmed cell deaths not occur but also cells, which normally differentiate into somatic tissues, instead undergo apoptosis. In addition independent of their lineage origin cell corpses persist in the embryo since many dead cells are not phagocytosed (Table I, Fig. 2a). Thus the reduction of CMD-1 activity can cause opposite effects. Normal apoptosis may be suppressed but also some cells, that would normally survive are driven into programmed cell death.

Opposite effects are also seen in cell cycle regulation. In 8 of 9 embryos we documented supernumerary cell cleavages in single or in combinations of the AB, MS, E and C lineages (Sulston et al., 1983) (Table I, Fig. 3). For example in the embryo *cmd-1* (2) all somatic lineages except D execute supernumerary cleavages. The D blastomere does undergo karyokinesis but not cytokinesis, which causes in the next cleavage a tetrapolar spindle, which resolves into two cells with two nuclei each. Cells contain varying numbers of nuclei (Fig. 2c) depending on whether later cytokineses succeed or fail. A similar phenotype has been reported in non-muscle

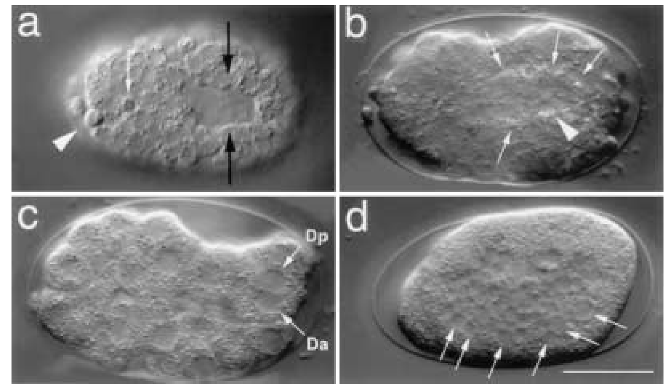


Fig. 2. Embryonic phenotypes observed with *cmd-1* RNAi. (**a**) Ventral Nomarski view of the *cmd-1* RNAi embryo (1) arrested at the premorphogenetic stage. The embryo showed defects in ventral closure (*black arrows*) and in apoptosis. Apoptotic cells resulting from either normal (ABalppppapp; *white arrow*) or abnormal cell death (ABarpapaap, which normally gives rise to neurons; *arrowhead*) were not engulfed (Sulston et al., 1983). (**b**) In the embryo *cmd-1* (3) the E-derived cells still show the typical characteristics of intestinal differentiation, although most execute an additional cleavage (*arrows*), i.e. cells are polarised and form the typical rhabditin granules. The *arrowhead* points at the cell Ealapp, which was produced by an additional cleavage. (**c**) Aberrant cleavage of the D blastomere in embryo *cmd-1* (2). The cell did not complete cytokinesis, which results in a cell containing two nuclei (*arrows* labelled Da and Dp, which correspond to the normal daughters of D). (**d**) This cytokinesis defect is also seen in *nmy-2* RNAi embryos, which develop into a single multinucleated cell. Some nuclei are indicated by *arrows*. Bar 20 μm.

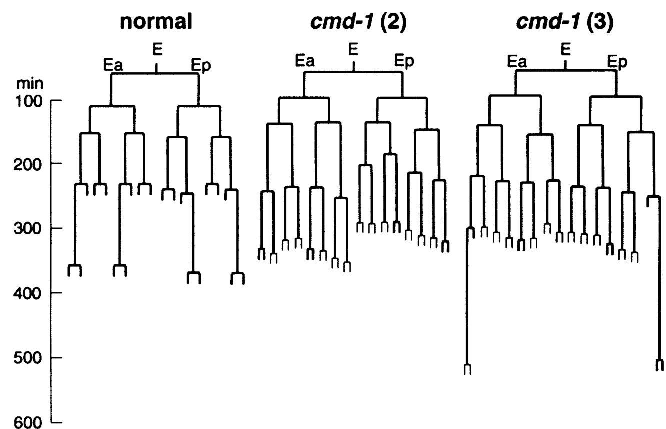


Fig. 3. Proliferation of intestinal cells in two *cmd-1* RNAi embryos. The figure shows lineage graphs of a normal E lineage (N2), which produces 20 intestinal cells during embryogenesis (*bold lines*) (Sulston et al., 1983) and the E lineages of the *cmd-1* RNAi embryos (2) and (3). In the RNAi embryos both E lineages generated 32 cells, however, the cells are produced in slightly different cleavage patterns (*thin lines*).

Tab. I. Phenotypic analysis of *cmd-1* RNAi embryos.

a	Embryo	Cell division in E	Cell division in other lineages	Phagocytosis	Ventral closure	Early gastrulation
	1	N	+ AB	–	–	–
	2	+	+ AB, MS, C	–	–	–
	3	+	+ AB, C	–	–	–
	4	+	n.d.	–	–	–
	5	+	n.d.	–	–	–
	6	+	n.d.	–	–	–
	7	1)	1)	n.d.	n.d.	–
	8	+	n.d.	–	–	–
	9	+	– AB	–	–	–
b	CD #	Birth	Death	Phagocytosis		
	1 (ABalaapapa)	208	262	435*		
	2 (ABalaappaa)	208	238	270		
	3 (ABalapapaa)	199	224	239		
	4 (ABalappaaa)	229	269	435*		
	5 (ABalppaaaa)	200	–	–		
	6 (ABalppaapa)	197	–	–		
	7 (ABaraaaapp)	228	260	435*		
	8 (ABarpaaapp)	190	283	435*		
	9 (ABplpappap)	194	228	248		
	10 (ABplppaaap)	226	275	435*		
	11 (ABplpppapp)	194	227	435*		
	12 (ABprppaaap)	192	232	326		
	13 (ABprpppapp)	177	–	–		
c	ABarpapaap	212	303	435*		
	ABarpapapp	218	327	435*		
	ABplppapap	199	255	435*		

(**a**) The phenotypes of the 9 embryos analysed with the 4D-microscope system are listed. The lineage of embryos (1) and (2) have been completely determined up to the premorphogenetic stage. The lineages of the other embryos were partially determined to test whether the phenotypes are typical. Columns "Cell division": N normal cell divisions, + additional cell divisions, – missing cell divisions. 1) In embryo (7) cell divisions arrested after the 8th cleavage division, therefore only the early gastrulation, which occurs before, but not the other phenotypes could be determined. Other columns: – defect, n.d. no data. (**b**) The 13 early cell deaths in embryo (1). Three normal CD survived (–) but three additional cell deaths occurred, which are listed in (**c**). Cell deaths, which are not engulfed until 435 min, which corresponds to the end of the recording are indicated (*). In normal embryogenesis engulfment occurs 15 ± 7 min after corpses are formed. In *cmd-1* RNAi embryos engulfment occurs at 123 ± 65 min after corpses are formed ($p < 0.001$). We assumed that corpses, which were not engulfed until the end of the recording were engulfed immediately afterwards. Since *cmd-1* RNAi embryos develop slower than normal embryos we also normalised the timing by multiplying by a factor of 0.77. In the other embryos, except no. 7, out of the 23 cell deaths we lineaged, 12 corpses were not engulfed. In embryo no. 8 we identified two ectopic cell deaths.

myosin 2 (*nmy-2*) RNAi embryos (Shelton et al., 1999) (Fig. 2d).

In addition, as can be seen in the three-dimensional representations of the nuclei of the two fully reconstructed *cmd-1* RNAi embryos (Fig. 4, supplementary information at http://www.tu-bs.de/institute/genetik/schnabel/refs.html), cells are not properly placed during embryogenesis, and in particular the descendents of ABarp fail to form the left-right symmetric y-shaped structure always formed during normal embryogenesis (Schnabel et al., 1997; supplementary information at http://www.tu-bs.de/institute/genetik/schnabel/refs.html). The earliest observed "migration" defect is the aberrant initiation of gastrulation, which is like the general migration defect also seen in non muscle myosin 1 (*nmy-1*) RNAi (F52B10.1) embryo (Fig. 4d, supplementary information at http://www.tu-bs.de/institute/genetik/schnabel/refs.html).

Calmodulin RNA interference through feeding

After our analysis was complete Kamath et al. (2003) reported an elegant high-throughput RNAi feeding analysis of almost all *C. elegans* genes. To our surprise they did not observe embryonic lethality for CaM and reported instead a mild postembryonic phenotype with slow growth and reduced fertility. In order to explore the striking difference between our microinjection results and the feeding results of Kamath et al. (2003) we cloned the *cmd-1* cDNA into the feeding vector L4440 and used it in *E. coli* RNAi feeding experiments according to the standard protocol (see Materials and methods).

Our RNAi feeding experiments essentially confirm those of Kamath et al. (2003). We observe embryonic lethality in only 8% of the progeny and find in another 8% an L1 arrest. A very slow growth was found for 18%. The remaining worms developed normally into fertile adults. Of these about one half laid only between 1 to 20 embryos ($n = 25$). The striking difference between the two delivery protocols is discussed below.

Discussion

Search of the *C. elegans* database identified a single CaM and four CaM-like genes in the nematode genome. The CaM-like proteins share with each other and with CaM 54 to 68% sequence in the core domain and have unusual N-terminal extensions not present in CaM (Fig. 1). The four EF hand sequences seem suitable for calcium binding except for the last

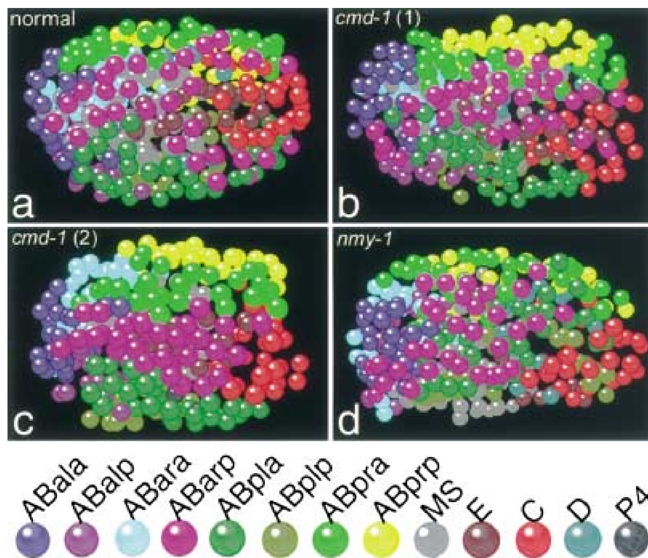


Fig. 4. Three-dimensional representations of normal and cmd-1 RNAi embryos (Schnabel et al., 1997). The spheres show the positions of the nuclei of a normal (**a**) and two *cmd-1* RNAi (**b**, **c**) embryos at the premorphogenetic stages (approximately 400 cells), as they were marked with SIMI°Biocell© in the corresponding 4D-recordings. To show the distribution of the descendants of the different lineages, the spheres were colour coded at the 12-cell stage embryos (code shown at bottom). All embryos are shown in a dorsal view, anterior to the left. In both *cmd-1* RNAi embryos the cells are not placed properly. For example the descendants of ABarp do not form the typical y-shaped configuration of cells. A *nmy-1* (non-muscle myosin 1) RNAi embryo (**d**) shows similar defects in cell positioning and gastrulation as the *cmd-1* embryos. The descendants of the MS founder cell (grey) are also not internalised. This suggests that the migration defects in the *cmd-1* RNAi embryos may be mediated through the Ca^{2+}-dependend regulation of non-muscle myosin.

hand in CAL-4. Amplification by PCR shows that all 5 genes are represented in a cDNA library from a mixed population of worms. The presence of only a single CaM gene in contrast to at least three nonallelic genes in vertebrates (Fischer et al., 1988) invited a functional analysis by RNA interference.

RNAi experiments, based on the microinjection delivery protocol, document that CaM is essential for embryonic development of *C. elegans*. Some defects seen with *cmd-1* RNAi embryos, such as the supernumerary cell divisions or the alteration of the pattern of apoptosis, could be due either to specific functions of CaM in the corresponding processes or to fate alterations of the affected cells. Although we cannot specifically exclude that the affected cells have an altered fate, this appears unlikely since the general differentiation pattern of cells (Sulston et al., 1983) is normal and in the case of the additional cleavages in the intestinal E lineage, cells still differentiate into intestinal cells (Fig. 2b). Thus *cmd-1* RNAi embryos show a wide range of phenotypes reflecting at least some of the diverse functions of calcium/CaM in the regulation of cellular processes. The "mosaicism" of the defects could be explained by the fact that the RNAi technique does not always completely eliminate the gene activity, so that in different embryos, or even in different cells of the same embryo, the activity may or may not fall below the different activity thresholds necessary for different processes.

Some defects such as problems in cell movement and gastrulation can be phenocopied in RNAi experiments with NMY-1 (non-muscle myosin 1) and others such as those in cytokinesis with NMY-2 (non-muscle myosin 2). These phenotypes may thus reflect the function of calmodulin in regulating the activity of non-muscle myosins (Guo and Kempheus, 1996; Shelton et al., 1999) directly or by means of kinases (Reiner et al., 1999; Eto et al., 1999; Raveh et al., 2001) and phosphatases (Tan et al., 2001; Bandyopadhyay et al., 2002), which are proposed to be CaM-dependent in *C. elegans* and in vertebrates (for recent reviews see (Snedden and Fromm, 1998; Chin and Means, 2000; Hoeflich and Ikura, 2002)). Moreover, CaM may also be involved in regulation of cellular movement through extracellular proteins such as the netrins which modulate branching and migrations of *C. elegans* axons in the CaM kinase II-dependent manner (Wang and Wadsworth, 2002) (for review on cell migration see (Montell, 1999)).

The opposing cell death activity we observed has also been described for the main coregulators of apoptosis in *C. elegans*, the proteins CED-9 and CED-4. Therefore it remains to be determined whether CaM operates together with these two proteins and the proteins EGL-1, CED-3 in the basic apoptotic machinery (for recent reviews see (Metzstein et al., 1998; Hengartner, 2001)) or regulates programmed cell death in a caspase-independent fashion. Interestingly, the last phenomenon has been observed recently for the human kinase DAP, which is modulated by CaM and participates in a wide range of apoptotic systems (Raveh et al., 2001; Raveh and Kimchi, 2001). Finally, involvement of CaM in the engulfment of apoptotic cells indicates the possibility that this protein may be one of the missing links in the engulfment pathway which already contains 6 different genes in *C. elegans* (Hengartner, 2001; Hoeppner et al., 2001).

However, there are several additional points which should also be discussed in the context of the CaM phenotype. *1)* The fact that CaM RNAi is lethal suggests that none of the CaM-like genes can substitute for CaM but by no means proves this claim. We know that CAL-1, CAL-2 and CAL-4 are expressed in adults (our unpublished results) but do not know so far whether these proteins are also present at the embryo stages of development when CaM depletion is lethal. *2)* In all unicellular organisms analyzed so far (see Introduction) CaM is essential for progression through the cell cycle. Thus, it would be a truly remarkable finding if the *C. elegans* embryo could undergo multiple rounds of cell division in the absence of CaM. A more plausible interpretation of this result is that RNAi did not sufficiently depleted CaM activity and that our phenotype also suffered to some extend from the same maternal pool as described in *Drosophila* by Heiman et al. (1996). Thus, the complex phenotype of the *C. elegans* RNAi embryos probably reflects a gradual loss of CaM function as opposed to a true genetic null.

In contrast to the embryonic lethality of CaM RNAi using delivery by microinjection (see above), delivery of the dsRNA by feeding does not allow an efficient silencing of the CaM gene. Kamath et al. (2003) report a mild postembryonic phenotype with slow growth and reduced fertility after RNAi feeding and we obtained similar results in our feeding experiments. Such a striking difference in phenotypes between the two RNAi delivery methods is not without precedence and conforms with the earlier generalisations made by Fire and coworkers. They showed that delivery by feeding can miss early phenotypes established by microinjection. This may be due to the fact that

the embryonic period is temporarily isolated from dsRNA delivery because of the impermeability of the eggshell (Timmons et al., 2001). In our experiments microinjection provided a 95% embryonic lethal phenotype for CaM RNAi while RNAi by feeding showed that only 8% of the embryos were affected. On the other hand RNAi by feeding can be more effective than microinjection to detect late developmental phenotypes (Timmons et al., 2001). Thus it seems that use of both delivery protocols can be useful. Based on our results for calmodulin and the report of Timmons et al. (2001) we expect that the feeding protocol used in the recently reported RNAi analysis of almost all *C. elegans* genes (Kamath et al., 2003) can miss some early developmental phenotypes.

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