MECHANISMS OF DEVELOPMENT 126 (2009) 382–393

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MECHANISMS OF DEVELOPMENT

Two cytochrome P450s in *Caenorhabditis elegans* are essential for the organization of eggshell, correct execution of meiosis and the polarization of embryo

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ARTICLE INFO

Article history:
Received 7 November 2008
Received in revised form
30 January 2009
Accepted 2 February 2009
Available online 11 February 2009

Keywords:
Caenorhabditis elegans
Cytochrome P450
Eggshell
Polarity
PAR proteins
Osmotic balance

ABSTRACT

The role of lipids in the process of embryonic development of *Caenorhabditis elegans* is still poorly understood. Cytochrome P450s, a class of lipid-modifying enzymes, are good candidates to be involved in the production or degradation of lipids essential for development. We investigated two highly similar cytochrome P450s in *C. elegans*, *cyp-31A2* and *cyp-31A3*, that are homologs of the gene responsible for Bietti crystalline corneoretinal dystrophy in humans. Depletion of both cytochromes either by RNAi or using a double deletion mutant, led to the failure of establishing the correct polarity of the embryo and to complete the extrusion of the polar bodies during meiosis. In addition, the egg became osmotic sensitive and permeable to dyes. The phenotype of *cyp-31A2* or *cyp-31A3* is very similar to a class of mutants that have polarization and osmotic defects (POD), thus the genes were renamed to *pod-7* and *pod-8*, respectively. Electron microscopic analysis demonstrated that the activity of *pod-7/pod-8* is crucial for the proper assembly of the eggshell and, in particular, for the production of its lipid-rich layer. Using a complementation with lipid extracts, we show that POD-7/POD-8 function together with a NADPH cytochrome P450 reductase, coded by *emb-8*, and are involved in the production of lipid(s) required for eggshell formation.

1. Introduction

The early stage of *Caenorhabditis elegans* embryo development is a well-orchestrated sequence of events that results in the first asymmetric division. The initial polarization of the embryo is required to execute the invariant plan of the development where a regulated order of cell divisions will produce a diversity of cell fates and, consequently, of tissues. The events preceding the first asymmetric division include the determination of the cell division plane, the formation of polarized cortical domains and the subsequent polarization of the cytoskeleton and cytoplasm (Cowan and Hyman, 2004).

The plan for an asymmetric division is already laid down by the entry of the sperm into the oocyte (fertilization). The sperm sends a signal to the female pronucleus, causing the resumption of meiosis, previously arrested in prophase I (Miller et al., 2001). The pronucleus of the oocyte goes through two anastral meiotic segregations and extrudes two polar bodies from the embryo cortex. The first polar body is localized at the anterior pole of the egg, whereas the second one stays associated with the embryo. Coincident with the

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0925-4773/$ - see front matter
doi:10.1016/j.mod.2009.02.001

completion of meiosis, about 20 min after fertilization, the process of polarization becomes visible. One of the manifestations of the polarization process is the redistribution of PAR proteins to the anterior or posterior cortexes of the embryo. Here, the cytoskeleton re-distributes the PAR proteins by movements that create membrane "ruffles": ingressions visible throughout the surface of the cortex. Proteins PAR-6, PKC-3 and PAR-3 form a trimeric complex and localize to the anterior pole of the embryo. This trimeric complex actively restricts proteins PAR-2 and PAR-1 to the posterior pole (Cuenca et al., 2003; Etemad-Moghadam et al., 1995; Hung and Kemphues, 1999; Tabuse et al., 1998; Watts et al., 1996). As Hao et al. showed, PAR-2 is a target of PKC-3 and is excluded from the anterior cortex by PKC-3 dependant phosphorylation (Hao et al., 2006). In turn, cortical PAR-2 prevents PAR-3/PAR-6/PKC-3 from returning to the posterior.

Generally, the role of proteins in the development of *C. elegans* is well recognized: between 100 and 400 maternal proteins are required for the first mitotic division (Gonczy et al., 1999). In contrast, the identity and the function of lipids required for the development are inadequately studied. It has been reported that mutations in genes involved in fatty acid production (*pod-2* and *fasn-1*, encoding enzymes acetyl-CoA carboxylase and fatty acid synthase respectively) cause a symmetric first cell division (Rappleye et al., 2003). These genes are members of a broad class of POD mutants (polarization and osmotic defects) that have been implicated in the maintenance of the osmotic balance in embryos. The permeability barrier of *C. elegans* embryos is likely to be largely mediated by the eggshell, a chemically resistant material that surrounds and isolates the embryo from the outer world. EM studies, performed mostly with parasitic nematodes, showed that the eggshell is a structure with three layers: vitelline, chitin and lipid-rich layers (Foor, 1967). Among other proteins, the POD genes contain enzymes involved in the production of chitin, an essential constituent of the eggshell (Johnston et al., 2006; Zhang et al., 2005).

As a starting point for our investigations, we decided to study the role of cytochrome P450s (CYPs), a class of enzymes that produce and modify lipids, in development. Genomic analysis of the nematode *C. elegans* revealed the existence of 83 CYPs. Simultaneous depletion of two of them, CYP-31A2 and CYP-31A3, caused embryonic lethality. The absence of these proteins resulted in osmotic sensitivity of the embryo and defects in polarization. In addition, meiosis failed: extrusion or the localization of polar bodies was incorrect, leading to polyploidy. Electron microscopic analysis demonstrated that embryous depleted of CYP-31A2 and CYP-31A3 possess a defective eggshell. Our data indicate that these two cytochrome P450s act together with EMB-8, a NADPH cytochrome P450 reductase to produce lipid(s) required for proper eggshell formation.

2. Results

2.1. *cyp-31A2/A3(RNAi) embryos fail to establish polarity and divide symmetrically*

CYP-31A2 (ORF H02I12.8) and CYP-31A3 (ORF Y17G9B.3) are members of the cytochrome P450 family 4. Members of this family are enzymes involved in the hydroxylation of lipids, in particular sterols or fatty acids (Werck-Reichhart and Feyereisen, 2000). CYP-31A2 and CYP-31A3 share 92% identity of their mRNA sequence and are almost identical on the peptide level. They also have a remarkable homology to the human CYP-4V2 (65% similarity and 45% identity) that has been linked to a severe disease: Bietti crystalline corneoretinal dystrophy (Lin et al., 2005). This disease, a form of night-blindness, is characterized by the accumulation of unidentified crystalline inclusions (presumably containing fatty acids) in the retina.

Genome-wide RNAi screens have shown that silencing of the *cyp-31A2* is embryonic lethal (Piano et al., 2000). It should be noted, however, that CYP-31A2 or CYP-31A3 could not be discriminated and it is not clear the depletion of which enzyme was responsible for the phenotype: the construct for the RNAi should silence both. Therefore, we will refer to *cyp-31A2/A3(RNAi)*. In our experiments, mothers fed with bacteria producing dsRNA against *cyp-31A2/A3* developed normally, but 100% of laid eggs never reached the onset of gastrulation and consequently did not hatch (not shown). In order to analyze stages of development affected by RNAi, we performed time-lapse microscopy of embryos derived from animals depleted of CYP-31A2/A3 by RNAi. One of the defects detected in time-lapse images was reduced contractility of the cortex. The ruffles normally observed in wild-type embryos (Fig. 1A; arrowheads; Suppl. movie 1) were very shallow or completely undetectable in *cyp-31A2/A3(RNAi)* embryos (Fig. 1C; Suppl. movie 2). Moreover, the pseudocleavage furrow, a transient ingression of the plasma membrane that occurs during the pronuclear migration, was often absent (25 out of 27 cases in *cyp-31A2/A3(RNAi)*; Fig. 1, compare middle panels in a and c, white arrow). Interestingly, this phenotype resembled that of embryos treated with the actin cytoskeleton-disrupting drug cytochalasin (Strome and Wood, 1983), *nmy-2(RNAi)* and *mlc-4(RNAi)* (Guo and Kemphues, 1996; Shelton et al., 1999). In contrast, processes that are regulated by microtubules, such as migration of the pronuclei and the rotation of the nuclear-centrosomal complex, were comparable with the wild-type situation. Thus, the depletion of CYP-31A2/A3 might influence activity connected to the actin cytoskeleton.

Polarization of the *C. elegans* embryo along its anterior–posterior axis is one of the decisive events in its development. It is known that the actin-cytoskeleton is directly involved in the polarization process. We examined the distribution of the well-established polarization markers, PAR-6 and PAR-2 proteins, in *cyp-31A2/A3(RNAi)* embryos. Reduction of CYP-31A2/A3 leads to a uniform cortical localization of PAR-6 (13 out of 16 cases in *cyp-31A2/A3(RNAi)*; Fig. 1D; Suppl. movie 2), instead of its normal anterior localization (Fig. 1B; Suppl. movie 1). The effect on PAR-2 is similar but relatively weaker: it accumulates at the posterior cortex in a reduced, small patch (11 out of 13 cases in *cyp-31A2/A3(RNAi)*; Fig. 1F; Suppl. movies 3 and 4), or alternatively, is mislocalized at the lateral edge of the embryo (not shown).

In half of CYP-31A2/A3-depleted embryos (13 out of 27 embryos) the first cell division generated two cells of roughly equal size and thus led to a symmetric division. Rest of the embryos displayed an asymmetric first division (14 out of

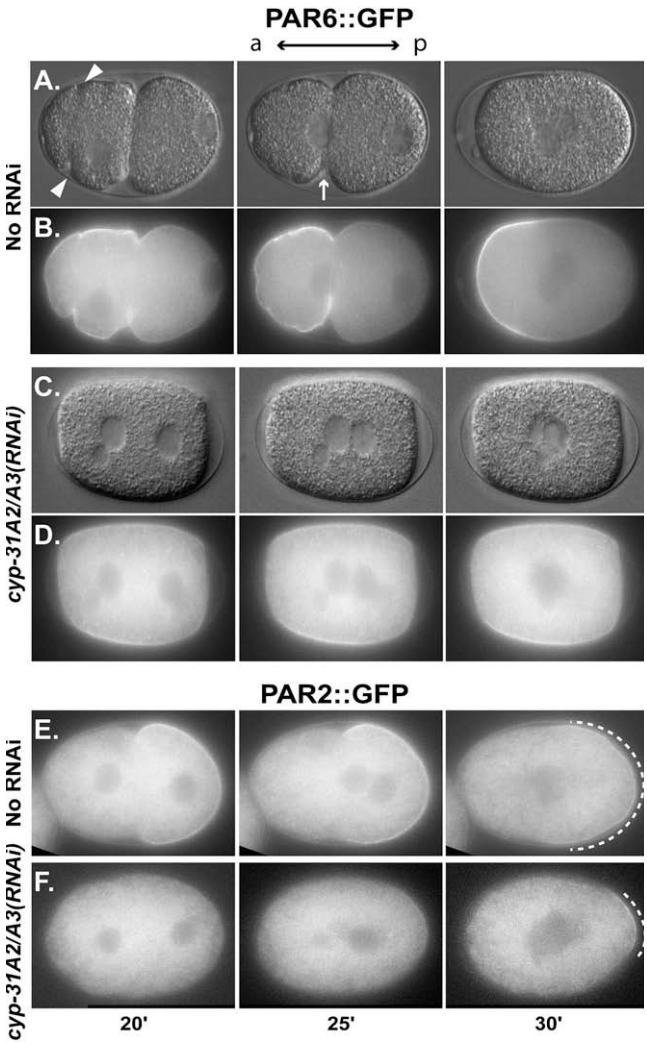

Fig. 1 – *cyp-31A2/A3(RNAi)* embryos fail to establish correct anterior-posterior polarity. Images show embryos at around 20, 25 and 30 min after the fertilization. A, C are DIC and B, D–F are fluorescent images. In non-treated embryos, PAR6::GFP is restricted to the anterior pole (B) whereas in *cyp-31A2/A3(RNAi)* embryos, PAR6::GFP localized in a symmetric manner throughout the cytoplasm and entire cortex (13 out of 16 embyos; D). Membrane ruffles or the pseudocleavage (white arrowheads and white arrow in (A), respectively) in embryos depleted of CYP-31A2/A3 are missing (25 out of 27 embryos; C). In wild-type embryos PAR2::GFP is restricted to the posterior pole (E). In *cyp-31A2/A3(RNAi)* embryos, PAR2::GFP is localized in the posterior side of the embryo but it fails to expand, resulting in a little patch (dashed line) (11 out of 13 embryos; F).

27) and all embryos arrested their development before the onset of the gastrulation after about 100 min.

2.2. *Embryos depleted of CYP-31A2/A3 are osmotic sensitive*

In addition to defects in the establishment of polarity, time-lapse images of CYP-31A2/A3-depleted embryos revealed other anomalies. They appeared swollen and filled the entire perimembrane space (the space between the plasma membrane of the embryo and the eggshell, see bellow). Moreover, many embryos burst during mounting for microscopic investigation.

Normally, the eggshell isolates the embryo from the external environment, preventing changes in the surrounding milieu from affecting development. The eggshell of *C. elegans* is resistant to mild acidic or basic treatments and is impermeable to water (Bird and Bird, 1991). In contrast, embryos with a defective eggshell (see later) placed in a hypotonic media respond by absorbing water, leading to the enlargement of the cell volume and a corresponding dilution of the internal concentration of ions. The defective eggshell cannot resist the resulting hydrostatic pressure and bursts. Thus, the swelling of embryos might be the result of eggshell damage or permeability. Interestingly, the polarity defects described above could not be rescued if *cyp-31A2/A3(RNAi)* embryos were placed in osmotically balanced medium such as EGM (Edgar's growth medium) (Edgar, 1995).

To test the permeability of *cyp-31A2/A3(RNAi)* eggs, we immersed them in solutions with dyes, such as Hoechst 33342 (stains DNA) or FM 4-64 (marks the membranes), that do not cross the barrier of the wild-type eggshell (Fig. 2A; the observed staining of the first polar body is discussed later, see below). In *cyp-31A2/A3(RNAi)* embryos, dyes diffused through the eggshell: Hoechst 33342 stained the DNA of the polar bodies and the nuclei, and FM 4-64 stained membranes and also some punctuate structures (most probably endosomes) (Fig. 2B). These results demonstrate that the eggshell in *cyp-31A2/A3(RNAi)* embryos is permeable.

The phenotype described so far implies that CYP-31A2/A3 should act in the embryo/eggs. We investigated the expression pattern of one of these enzymes (CYP-31A3) by putting GFP under *cyp-31A3* promoter. As seen in Fig. 2E,F, the expression is confined to the gonads, oocytes and can be detected in eggs.

2.3. *CYP-31A2/A3 is required for immobilization of the 1st PB and extrusion of the 2nd PB*

An additional trait found in *cyp-31A2/A3(RNAi)* embryos was polar body defects during meiosis (detected in about 86% of the embryos). After fertilization, the egg nucleus completes two rounds of meiotic divisions and after each division extrudes one polar body from the embryo. The first polar body, resulting from meiosis I, is separated from the embryo plasma membrane and becomes immobilized in the pole of the egg. The second polar body, resulting from meiosis II, remains attached to the surface of the embryo. Although the first polar body was extruded, it was not localized at the anterior pole as in the wild-type egg (Suppl. movie 5) but remained associated with the embryo (Suppl. movie 6). Most often, the second polar body was not extruded. The resulting two female pronuclei behaved normally, migrating towards the male pronucleus and fusing their DNA contents, thus altering the ploidy of the embryo (Fig. 2D; compare Suppl. movies 5 and 6).

At first glance, the three traits detected in *cyp-31A2/A3(RNAi)* embryos (osmotic sensitivity, symmetric cell division and failure of extrusion of a polar body) seem to be related

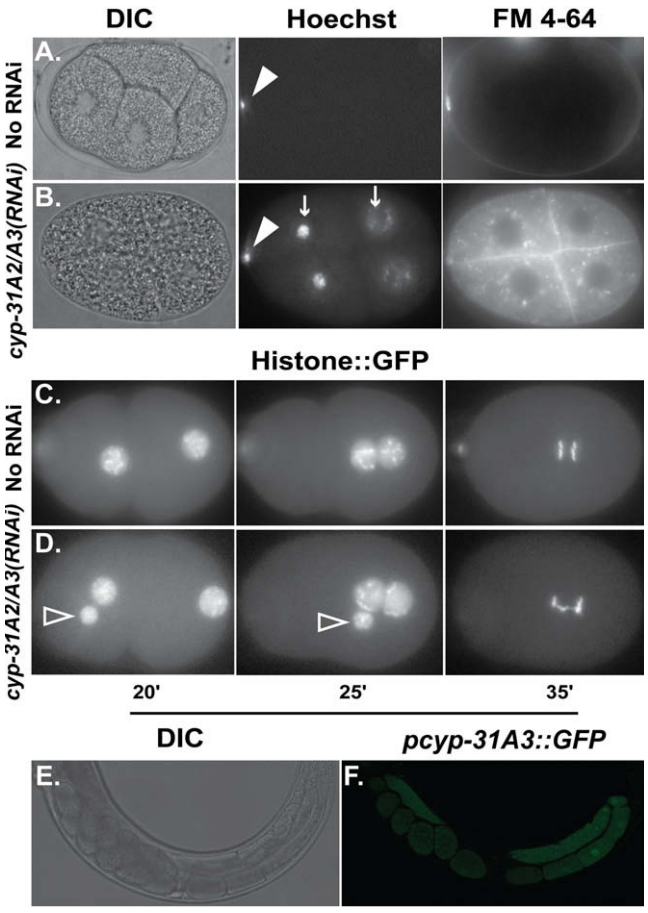

Fig. 2 – *cyp-31A2/A3*(RNAi) embryos are permeable to dyes and fail to complete the extrusion of one of the polar bodies. Hoechst 33342 and FM 4-64 cannot penetrate the impermeable eggshell of a wild-type embryo (A). Note the staining of the 1st polar body (white arrowhead). The eggshell of *cyp-31A2/A3*(RNAi) embryos is permeable to these dyes: Hoechst 33342 stains DNA of the polar bodies and of the nuclei (white arrows), and FM 4-64 stains membranes and also some punctuate structures (B). In non-treated embryos of the histone::GFP line, polar bodies are extruded at the anterior pole (C). In *cyp-31A2/A3*(RNAi) embryos, however, one of the polar bodies eventually fuses with the zygotic nucleus (D, empty arrowhead). *cyp-31A3* is expressed in the gonads, oocytes and embryos (E, DIC image; F, GFP fluorescence).

to different cellular processes that should exploit diverse protein machineries. However, a number of single gene mutants or RNAi-knockdowns, has been reported to display the set of similar phenotypes (Table 1). Six of these mutants are designated as POD (polarity and osmotic defects). We suggest renaming *cyp-31A2* and *cyp-31A3* into *pod-7* and *pod-8*, respectively.

2.4. A double deletion mutant of pod-7 and pod-8 has an identical phenotype to RNAi of these genes

While performing this study, deletion mutants of *pod-7* and *pod-8* became available. We tested whether the RNAi phenotypes described above were indeed the consequence of silencing these genes and not caused by secondary targets.

Individual mutants of *pod-7* and *pod-8* (*tm2711* and *tm3224*, respectively) displayed no visible phenotypes and embryos develop normally when laid on NGM agar (standard osmotic conditions). However, when the eggs were laid on agarose prepared in dd water (hypoosmotic conditions), they showed a slight osmotic sensitivity (Fig. 3, compare agarose with regular NGM for the single mutants). Thus, we decided to produce a double mutant. The phenotype of double mutants and RNAi phenotypes were similarly maternal embryonic lethal: the first generation grows normally, whereas laid eggs never hatch (Fig. 3). In addition, the embryos from double *pod-7*(*tm2711*) *pod-8*(*tm3224*) laid on agarose (hypoosmotic conditions) exploded (not shown).

2.5. pod-7/pod-8 embryos exhibit an abnormal morphology of eggshell

Osmotic sensitivity and permeability to dyes indicate that the barrier function of the eggshell in *pod-7/pod-8*(RNAi) and *pod-7*(*tm2711*) *pod-8*(*tm3224*) embryos is damaged. In order to find out whether these defects are manifested at the morphological level, we performed electron microscopic studies of the embryos (Fig. 4 and Suppl. Fig. 1).

The wild-type eggshell has a thickness of about 200 nm, although it is much wider at the poles, (see below) and three easily distinguishable layers (Fig. 4C; Bird and Bird, 1991; Rappleye et al., 1999). The most external vitelline layer (VL) has the appearance of a continuous sharp line; the underlying chitin layer (CL) could be seen as a light gray strip. The innermost electron dense stratum is called the lipid-rich layer (LrL). These three layers are followed by a perimembrane space (Fig. 4A) that, with irregular thickness, surrounds the plasma membrane of the embryo.

EM images of *pod-7(tm2711) pod-8(tm3224)* embryos showed quite a different picture (Fig. 4B and D). At low magnification (Fig. 4A and B) *pod-7(tm2711) pod-8(tm3224)* embryos appeared swollen and the perimembrane space was almost undetectable. At higher magnification (Fig. 4C and D), the vitelline layer appeared slightly fuzzier than in wild-type embryos. Layers beneath VL were not discernible. The dark lipid-rich layer was almost entirely absent and the chitin layer looked fuzzy and disorganized. Thus, the aberrations detected in *pod-7(tm2711) pod-8(tm3224)* embryos may be due to an inappropriate construction of the eggshell, in particular, the absence of the lipid-rich layer. Very similar EM images were obtained with *pod-7/pod-8*(RNAi) (Suppl. Fig. 1).

The EM data indicate that POD-7/POD-8 might be involved in the production of the lipid-rich layer of the eggshell. Could this be a reason for the permeability of POD-7/POD-8 depleted embryos? In order to answer this question we investigated the contribution of particular layers to the maintenance of the permeability barrier.

The vitelline layer can be removed from the egg by brief bleaching with sodium hypochlorite without affecting the further development of the embryo (Schierenberg and Junkersdorf, 1992). Embryos after bleaching are neither osmotic sensitive nor permeable to dyes (Fig. 5B). Coincident with synthesis of the eggshell, the first polar body is extruded from the

Table 1 – Genes displaying osmotic sensitivity, defects in polarization and incorrect execution of meiosis.

Gene	ORF	Function	Ref.
pod-1	Y72A2B.1	Coronin-like	Rappleye et al. (1999)
pod-2	W09B6.1	AcCoA carboxylase	Rappleye et al. (2003)
fasn-1	F32H2.5	Fatty acid synthase	
emb-8	K10D2.6	CYP reductase	
pod-7	H02I12.8	CYP	This study
pod-8	Y17G9B.3	CYP	This study
chs-1	T25G3.2	Chitin synthase	Johnston et al. (2006)
gna-2	T23G11	Glucosamine-6-P N-acetyltransferase	
cpg-1	C07G2.1	Chitin-binding	
cpg-2	B0280.5	Chitin-binding	
squ-5	T24D1.1	Chondroitin synthase	Olson et al. (2006)
APC/separase pathway			
pod-3	W10C6.1	APC subunit	Rappleye et al. (2002)
pod-4	F10C5.1	APC subunit	
pod-5	Y110A7A.17	APC subunit	
pod-6	F10B5.6	APC subunit	
emb-30	F54C8.3	APC subunit	
ubq-2	ZK1010.1	Ubiquitin	
fzy-1	ZK177.6	CDC-20	
sep-1	Y47G6A.12	Separase	
cyk-3	ZK328.1	Ubiquitin hydrolase	Kaitna et al. (2002)

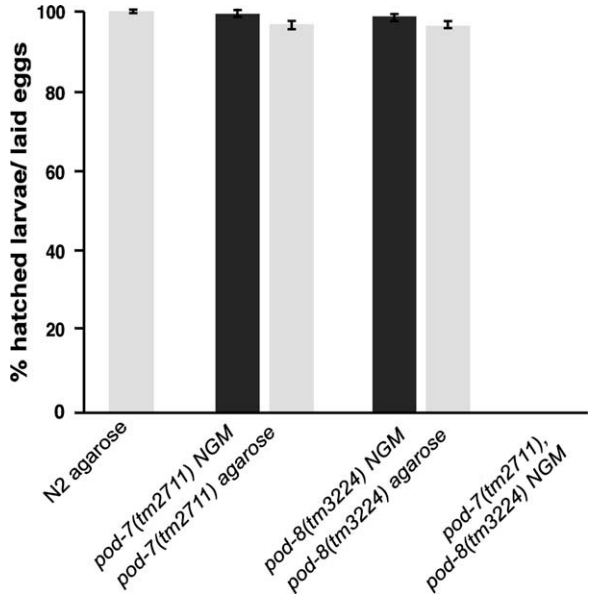

Fig. 3 – Osmotic sensitivity of single mutants *pod-7(tm2711)* *and pod-8(tm3224)*, *and double pod-7(tm2711) pod-8(tm3224)*. Eggs were laid either on NGM agar (standard osmotic conditions) or on agarose prepared in ddH_2O. The development of eggs was monitored after 24 h.

plasma membrane and localized at the anterior pole. This polar body can be stained in intact embryos by Hoechst 33342 (Fig. 5A), but is lost after the treatment with hypochlorite (Fig. 5B), suggesting that the VL maintains the 1st PB within the egg.

The chitin layer can be digested by treatment with chitinase after the bleaching (Wolf et al., 1983). The CL confers shape to the egg: after removal of the chitin layer, the embryo becomes rounded (Fig. 5C) and fragile. After the procedures with hypochlorite and chitinase, only the lipid-rich layer should be left surrounding the embryos. Nevertheless, embryos are not stained by dyes (Fig. 5C). Note that fragments of gonads surrounding the embryo are strongly stained. Thus, the lipid-rich layer is the stratum that confers the impermeability of the eggs to dyes.

2.6. *The first polar body is immersed in the lipid-rich layer*

Our data suggests that POD-7/POD-8 is required for the production of the eggshell, specifically the lipid-rich layer. This layer is essential for providing a permeability barrier in the developing embryo and helps to maintain the osmotic balance of the egg. Furthermore, we have shown that *pod-7/pod-8(RNAi)* embryos are defective in maintaining the strict position of the 1st polar body within the eggshell. We wondered whether these two processes, eggshell production and 1st PB localization, were linked. Given that the first polar body in intact embryos was stained with Hoechst 33342 (Fig. 5A), it seemed that this polar body could not be beyond the impermeable lipid-rich layer. At the same time, the observation provides a clue for the temporal relationship between the execution of meiosis and the formation of the eggshell.

Normally, the first polar body is localized on the anterior apex of the egg (Fig. 6A, DIC image). Inspection of serial sections of the embryo (more than 200 sections have been produced in five embryos) showed that the first polar body was associated with the chitin layer and was largely embedded in the lipid-rich layer (Fig. 6B). Note that the LrL at the site of the 1st PB is much thicker than observed in regular eggshell cross-sections (compare to Fig. 4A). We could not detect any opening in the eggshell near the polar body. The observed localization of the first polar body provides a likely explana-

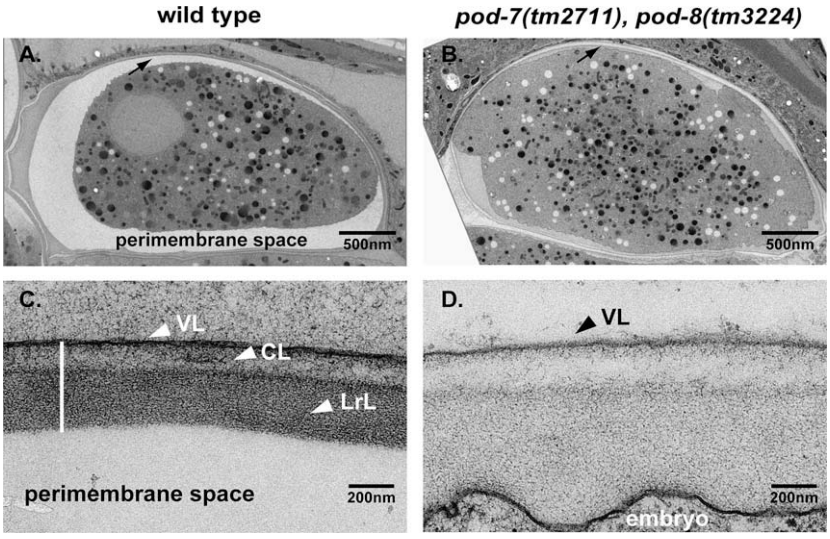

Fig. 4 – *pod-7*(*tm2711*) *pod-8*(*tm3224*) embryos have a defective eggshell. (A, B) EM low magnification (A, wild-type; B, *pod-7*(*tm2711*) *pod-8*(*tm3224*)), note the swollen embryo in B; arrows – eggshell region. (C, D) EM high magnification (C, wild-type; D, *pod-7*(*tm2711*) *pod-8*(*tm3224*)). Wild-type embryo shows a typical, well-separated three layered structure of the eggshell; VL, vitelline layer; CL, chitine layer; LrL, lipid-rich layer. *pod-7*(*tm2711*) *pod-8*(*tm3224*) display a disorganized arrangement of the layers. There is no clearly distinguishable LrL.

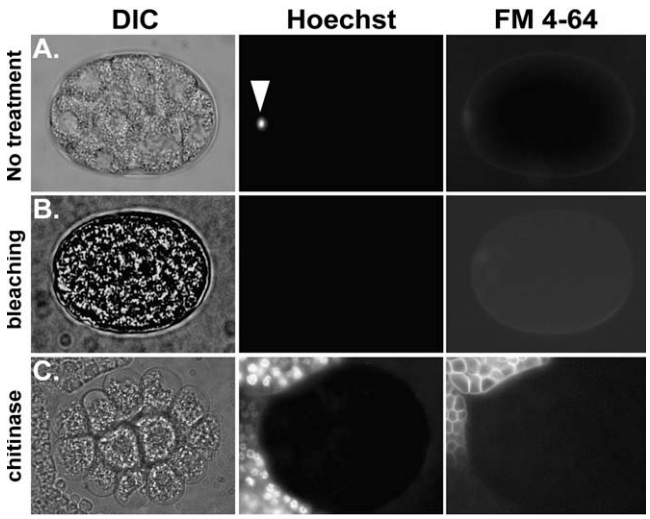

Fig. 5 – Role of individual layers of the wild-type eggshell in maintaining the permeability barrier. Wild-type embryo stained with Hoechst 33342 and FM 4-64 without any treatment (A), after bleaching (B), and subsequent chitinase-treatment (C). Only the first polar body is labeled (A). Note the loss of this polar body after bleaching (B). Despite the rounding up of the embryo after the chitinase-treatment, it still remains impermeable to dyes (C). In contrast, surrounding fragments of gonads are strongly stained.

tion as to why it can be stained by Hoechst 33342: the vitelline, as well as the chitin layers, are probably permeable to the dyes, whereas the LrL provides the dye permeability barrier but does not completely surround the 1st PB. The disappearance of the polar body after the bleaching may be explained by the partial destruction of vitelline and chitin layers by hypochlorite. Most important, however, is the temporal connection between the extrusion of the first polar body and the termination of the eggshell production. Given that the 1st PB is located between the chitin layer and the LrL, we suspect that the extrusion process occurs simultaneously with the formation of LrL or immediately precedes it. The absence of the LrL in *pod-7*/*pod-8*(RNAi) embryos may account for the inability of eggs to localize the first polar body to the anterior pole.

We also investigated the localization of the second polar body. Finding of the latter, however, was more complicated, because this polar body has no fixed position in the embryo. In four polar bodies analyzed, all were clearly separated from the embryo by membranes (Fig. 6C). Most surprising, however, was the finding that this polar body is immersed in a fibrous material (Fig. 6C, EmL). Moreover this material was found to surround the entire perimeter of the embryo (Fig. 6D; a segment of the lateral part is shown). The thickness of this layer is between 100 and 200 nm, except the place where the second polar layer is attached to the embryo. To the best of our knowledge, this layer was not described earlier. In the following, we will refer to it as the embryonic layer (EmL).

2.7. *The NADPH cytochrome P450 reductase, emb-8, and separase, sep-1, show a similar phenotype to pod-7/pod8 and have defective eggshells*

As mentioned above, mutations in several genes display a similar phenotype to *pod-7*/*pod-8* (Table 1). One of these, *emb-8*, encodes a NADPH cytochrome P450 reductase (NCPR) and thus could be functionally related to CYPs. NCPRs are supplying electrons to CYPs for their oxidative reactions (Gutierrez et al., 2003). A thermo-sensitive allele of *emb-8*(*hc69*), at a non-permissive temperature exhibits a phenotype identical to *pod-7*/*pod-8*(RNAi) (Rappleye et al., 2003). We asked whether

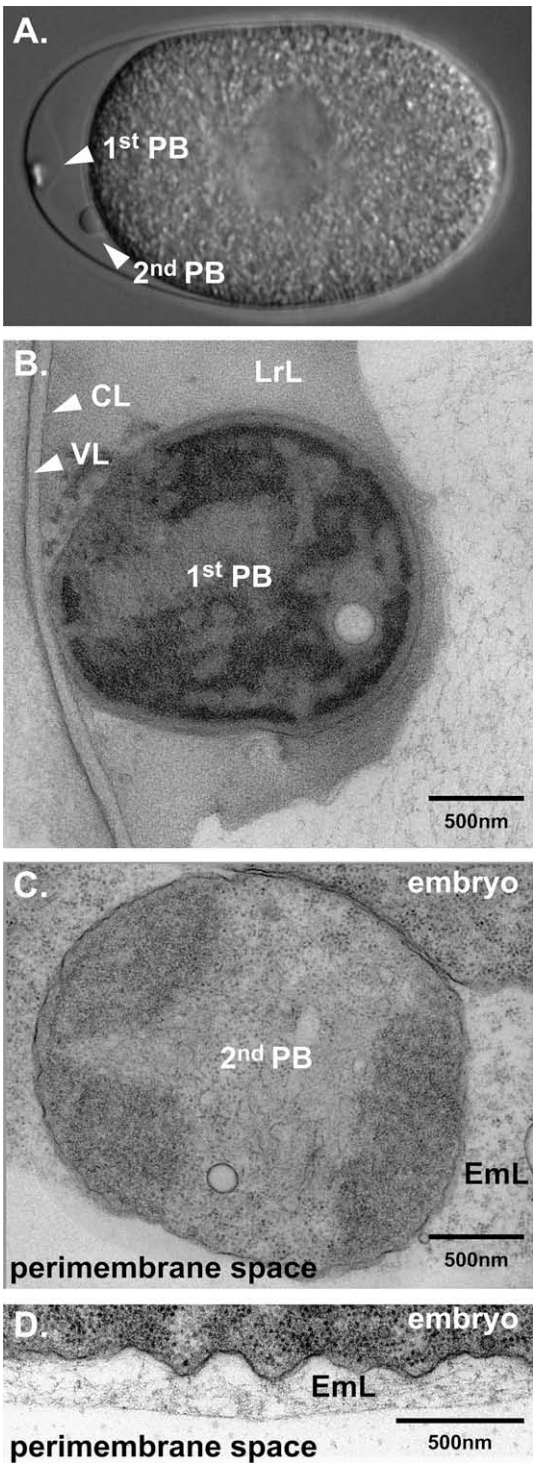

Fig. 6 – The two polar bodies in *C. elegans* embryos are differently localized after the extrusion. (A) DIC image of a wild-type embryo with two well visible polar bodies. The 1st PB is localized at the apex of the anterior whereas the 2nd PB stays in contact with its plasma membrane. (B) and (C), EM of sections. The 1st PB is tightly associated with chitin layer and is fully embedded in LrL (B). The 2nd PB is separated from embryo and is surrounded by a filamentous, embryonic layer (EmL) (C). The entire embryo is surrounded by EmL (D).

the eggshell of *emb-8* is also morphologically defective. Indeed, EM images of mutant embryos are very similar to *pod-7/pod-8*(RNAi) (Suppl. Fig. 1, compare panels B and C). The defects observed, however, are even more severe. The differences in the strength of the phenotype between *emb-8*(*hc69*) and *pod-7/pod-8*(RNAi) may be explained by the different efficiency of the protein depletion. Alternatively, EMB-8 as an NCPR might work with another CYP(s) required for eggshell formation.

As mentioned above, POD class includes genes responsible directly for correct execution of meiosis, such as subunits of APC and separase (Table 1; Bembenek et al., 2007; Rappleye et al., 2002; Siomos et al., 2001). Because mutants of these genes are all osmotic sensitive we asked whether their eggshell is also defective. As a representative of this group we chose *sep-1*(*e2406*) (Bembenek et al., 2007; Siomos et al., 2001). The EM images of the eggshell of *sep-1*(*e2406*) are very similar to that of *pod-7/pod-8*(RNAi) or *emb-8*(*nc69*) (Bembenek et al., 2007 and Suppl. Fig. 1D). Thus APC and separase might be involved in the regulation of eggshell formation which requires POD-7/POD-8 activity.

2.8. *POD-7/POD-8 and EMB-8 might be involved in the same metabolic pathway, producing a lipid required for the formation of the eggshell*

Defects in some genes involved in lipid metabolism can be rescued by the addition of specific lipids to the media (Entchev et al., 2008; Matyash et al., 2004; Rappleye et al., 2003; Watts and Browse, 2006). We decided to use this approach and to elucidate: (1) Can deficiency in POD-7/POD-8 be rescued by feeding of mothers with diverse lipidic extracts from wild-type worms? (2) Are POD-7/POD-8 and EMB-8 acting in the same pathway? (3) What is the chemical identity of the active lipids?

We set up a bioassay for testing and purifying lipids capable of rescuing the embryonic lethality caused by *pod-7/pod-8*(RNAi). Because the RNAi phenotype suggested that the metabolic product of *POD-7/POD-8* should be specifically required during embryonic development, we decided to use embryos as a source for extracts. In order to obtain high density of gravid adults we used *daf-22*(*m130*). This mutant does not make dauers because it has problems in production of dauer-inducing pheromone (Golden and Riddle, 1985). Otherwise it is wild-type in all aspects of development. The initial methanolic extract of embryos was further extracted with hexane, to devoid it of more hydrophobic molecules (producing Fractions A and B, respectively). When fed to *pod-7/pod-8*(RNAi) worms, Fraction A was able to rescue the embryonic lethality very effectively (Fig. 7). Fraction B was inactive (not shown). This suggests that the metabolic product(s) of POD-7/POD-8 is a rather hydrophilic lipid. As seen, *emb-8*(RNAi) worms can also be rescued by a lipid from *daf-22*(*m130*) extract.

Because POD-7/POD-8 and EMB-8 belong to functionally linked classes of CYPs and NCPRs, respectively, our data suggests that these two enzymes act together. To test this hypothesis, we prepared a lipidic extract from *emb-8*(*hc69*) worms grown at a non-permissive temperature and com-

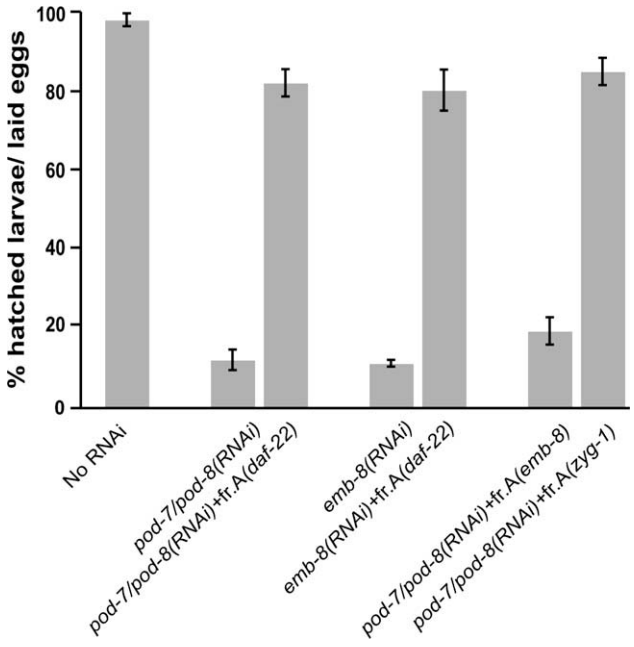

Fig. 7 – Methanol fraction (Fraction A) of extracts from control worms, but not from *emb-8(hc69)* mutant, can rescue the embryonic lethality caused by *pod-7/pod-8(RNAi)*. The rescue in the bioassay was quantified as the percentage of hatched larvae vs. total laid eggs. For a control extract, *daf-22(m130)* line was used. This line is dauer defective and allows producing large amount of worms but otherwise has a wild-type eggshell.

pared its activity with the wild-type extract. We reasoned that if two enzymes are acting together, then Fraction A from *emb-8(hc69)* worms should not contain the product of POD-7/POD-8 and consequently would be inactive. Indeed, the extract from mutant worms was not able to rescue the embryonic lethality of *pod-7/pod-8(RNAi)* (Fig. 7). An extract from another embryonic lethal mutant, *zyg-1(b1)*, that is not connected to lipid metabolism, was as active as the control extract. This data strongly suggests that EMB-8 might act in the pathway upstream or parallel to POD-7/POD-8. It must be noted that these proteins should be acting downstream of FASN-1 or ACC (POD-2) enzymes involved in the initiation of fatty acid synthesis and belonging to the POD class (Rappleye et al., 2003). Addition of either palmitic, stearic or oleic acids to the food could not rescue the embryonic lethality of *pod-7/pod-8(RNAi)* or *emb-8(hc69)* (not shown), suggesting that POD-7/POD-8 and EMB-8 act at a late stage of the biosynthetic pathway. In conclusion, this data place the NCPR EMB-8, along with the CYP POD-7/POD-8, in a pathway essential for the formation of a proper eggshell and in particular, the lipid-rich layer.

3. Discussion

Depletion of CYP-31A2 or CYP-31A3 (POD-7 and POD-8, respectively) or a NADPH Cytochrome P450 reductase (EMB-8) causes several anomalies that eventually lead to embryonic lethality: osmotic imbalance, incorrect execution of meiosis and impaired establishment of polarity. EM studies demonstrate that POD-7/POD-8, as well as EMB-8, are required for the formation of a functional eggshell. Moreover, the product of these two cytochrome P450s is a lipid since hydrophobic extract from embryos with active POD-7/POD-8 can rescue embryonic lethality of *pod-7/pod-8(RNAi)*.

How are processes such as eggshell formation, osmotic balance, execution of meiosis and embryonic polarity interconnected? What are the temporal and causal relationships between them? Some clues can be derived from a rough time-course of events that precede the first embryonic division (Fig. 8). The formation of the eggshell and the execution of meiosis start much earlier than cortical polarization. POD genes appear to act before PAR genes and there are many genes that influence the polarization but do not display any osmotic sensitivity or defects in meiosis (Gonczy et al., 2000; Guo and Kemphues, 1996; Shelton et al., 1999). A connection between structure of eggshell and execution of meiosis appears reasonable because the synthesis of eggshell and meiosis take place almost simultaneously: it would be efficient for the cell to coordinate both processes through the same regulatory machinery. As mentioned above, many POD genes are connected with the execution of meiosis. For instance, the meiotic regulatory complex APC^{cdc20} carries out a well-characterized role in the degradation of securin to activate separase at anaphase. Separase cleaves cohesins and eventually

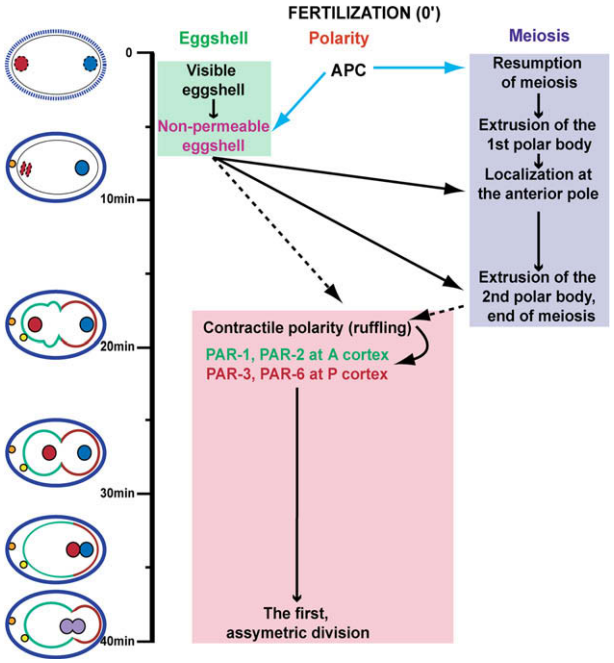

Fig. 8 – Timeline of the first embryonic division. The synthesis of the eggshell, execution of the meiosis and polarization are depicted in different colors. The first two start simultaneously, directly after the fertilization (point zero). The contractile and cortical polarizations occur later. On the left, positions of pronuclei and of residual bodies are shown. Red – female pronucleus, blue – male pronucleus. Solid arrows show observed causal connections; Dashed arrows indicate hypothetical connections.

resolves the chromosomes during meiosis. Recently it has been shown that during meiotic anaphase I separase resides on the cortical granules, exocytosis of which is required for eggshell formation (Bembenek et al., 2007). The components of the cell cycle machinery including separase are required for cortical granule secretion (Bembenek et al., 2007). Thus, the cell cycle machinery has a dual role in execution of meiosis and eggshell formation providing link and coordination between both processes. However, the connection between cortical granules and enzymes (such as CHS-1, SQV-4, POD-7, POD-8) in *C. elegans* eggshell formation remains to be clarified.

POD genes are most likely involved in more than one process upstream of cortical polarization. POD-7/POD-8, for instance, may be involved in the production of the components of the eggshell and in parallel regulate polarization via other substances. Very interesting in this context is a suggestion that *pod-7/pod-8* are genes involved in the production of a derivative of polyunsaturated fatty acids that attracts sperm to the oocyte (Kubagawa et al., 2006). Obviously, the characterization of the lipid product(s) of POD-7/POD-8/EMB-8 will be decisive for the clarification of these issues.

Our experiments reveal that embryos depleted of POD-7/POD-8 are unable to produce a lipid-rich layer and become permeable. On the other hand, after subsequent treatments with hypochlorite and chitinase embryos are fragile and very sensitive to sudden changes in salt concentration: when placed in a hypotonic medium (50 mM KCl), they respond by increasing cell volume until they explode (not shown). This observation indicates that the lipid-rich layer alone is not sufficient to ensure the correct regulation of osmotic balance. Moreover, two papers show that silencing of enzymes involved in the production of chitin, or proteins binding to chitin, have an identical phenotype to *pod-7/pod-8*(RNAi) in osmotic sensitivity and polarization (Johnston et al., 2006; Zhang et al., 2005). Thus, the eggshell appears to be a composite, where only correct assembly and interconnection of layers warrant its non-permeability to water/ions and hydrophobic dyes. The product of POD-7/POD-8 might be involved, not only in the formation of the lipid-rich layer, but also in assembly of the three layers.

According to our preliminary experiments an estimate of the abundance of the material that rescues POD-7/POD-8 in the total extract of embryos is very low. A challenging task for future biochemical analysis is the purification of eggshell. The homology to other CYPs indicates that POD-7/POD-8 could be a hydroxylase of a fatty acid or its derivatives. Indeed, Fraction A of the extract that rescues embryonic lethality should contain rather hydrophilic, hydroxylated-lipids (T.V.K., unpublished). As mentioned above, POD-7/POD-8 and EMB-8 should act on late stages of the pathway because embryonic lethality cannot be rescued either by individual fatty acids (palmitate, oleate, stearate, etc.) or a mixture of fatty acids extracted and partially purified from wild-type worms.

In summary, we show that the activity of POD-7/POD-8/EMB-8, in the production of lipids, is important for the development of the *C. elegans* embryo. The production of a proper eggshell, and in particular its lipid-rich layer, is a prerequisite for the correct execution of meiosis (localization of the 1st polar body and the extrusion of the 2nd polar body) and anterior–posterior polarization of the embryo. The identification of the chemical nature of the products of POD-7/POD-8 might facilitate the investigation of human CYP-4V2 and, thus, research on a medication, decelerating or preventing the process of crystal formation in the retina of Bietti crystalline corneoretinal dystrophy patients.

4. Materials and methods

4.1. *Worm strains*

Wild-type as well as mutant strains were routinely propagated on NGM agar plates (Brenner, 1974). The following strains were used: N2 Bristol (wild-type), *sep-1*(*e2406*) *I/hT2*[*bli-4*(*e937*) *let-?*(*q782*) *qIs48*] (*I;III*) (WH216, CGC), *daf-22*(*m130*) *II* (DR476, CGC), *zyg-1*(*b1*) *II* (DH1, CGC), *emb-8*(*hc69*) *III* (MC69, CGC), *rrf-3*(*pk1426*) *II* (NL2099, CGC) from *Caenorhabditis* Genetics Center (CGC), University of Minnesota, Minneapolis, USA).

The mutant deletion strains *cyp-31a2*(*tm2711*) and *cyp-31a3(tm3224)* were isolated in mutagenesis screens (Gengyo-Ando and Mitani, 2000) by the Japanese deletion consortium (National Bioresource Project for the Experimental Animal "Nematode C. elegans"). *cyp-31a2(tm2711)* was out crossed 4 times and *cyp-31a3(tm3224)* was out crossed 2 times with N2 wild-type. Double mutant *cyp-31a2(tm2711) cyp-31a3(tm3224)/nT1[qIs51] (IV;V)* were obtained by standard genetic methods with detection of the single deletions by PCR.

sep-1(*e2406*) *and cyp-31a2*(*tm2711*) *cyp-31a3*(*tm3224*) homozygous mutants were selected using a fluorescent dissecting microscope based on the absence of a GFP signal seen from the balancer *hT2* or *nT1*, respectively, in the heterozygous siblings. The following GFP transgenic strains: GFP::PAR-2, GFP::PAR-6 and GFP::H2B were a kind gift from Anthony Hyman (MPI-CBG, Dresden).

To quantify the embryonic lethality, L4 worms were placed on 12-well plates with either NGM agar or agarose 1%. After 24 h at 20 °C, adults were removed. After another 24 h at 20 °C, embryos and larvae were counted using a dissecting microscope.

4.2. *RNAi interference*

To perform RNAi by feeding, a portion of the H02I12.8 genomic DNA (0.85 Kb) was amplified by PCR with primers containing Not I linkers (primers *ccgcggccgctttccagcacctcaatcagcc* and *ccgcggccgcaatgcgagacgaccttccatc*). The PCR product was inserted into Not I site of L4440 DoubleT-7script II vector. RNAi by feeding was performed as described elsewhere (Fraser et al., 2000).

RNA-interference by microinjection: *cyp-31a2* was amplified from the L4440 DoubleT-7script II construct using the previously reported primers, linked to T7 and T3 RNA polymerases promoters. PCR products were purified using a PCR clean up kit (Qiagen), eluted in 40 μl of water and used as templates for 25 μl T3 and T7 in vitro transcription reactions (Ambion). After the transcription reaction, DNase (1.3 μl/reaction) was added and incubated for 15 min at

37 °C. The product of the reaction was cleaned using an RNeasy kit (Qiagen), and the RNA was eluted in a final volume of 60 μl. T3 and T7 reactions were pooled and mixed with 3× injection buffer (60 mM K_2HPO_4, pH 7.5, 9 mM K-citrate, pH 7.5 and 6% PEG 6000). After incubating the reaction at 94 °C for 10 min, annealing was achieved by incubation for 60 min at 37 °C. Samples of the T3 and T7 transcription reactions and the annealed dsRNA were loaded on an agarose gel. Annealed dsRNA exhibited a band shift compared to single stranded RNAs. DsRNA was aliquoted, snap frozen in liquid nitrogen and stored at −80 °C.

The dsRNA was injected into L4 or young adults, the animals were transferred to 25 °C and the dissected embryos were observed in the microscope after 24 h.

4.3. *Generation of cyp-31A3::GFP-transcription reporter*

pcyp-31A3::GFP worm strains were generated by ballistic transformation of *unc-119(ed3)* with modified fosmid WRM0636CA01 (Genome Sciences Centre, Canada) in which the coding sequence of *cyp-31A3* was replaced by EGFP cassette. This modified fosmid was produced by recombineering method (described in (Sarov et al., 2006)). egfp-kan cassette was amplified with primers tttgctcgttttttcgctcaaataaattattt cagaaggttttgcaatgagctcaggaggtagcgg and catgcattaaaaatata taatcatcaagaataaaattggctattttgaaattaggcagatcgtcagtcag. 18 independent lines of transformed worms were identified based on uncoordinated phenotype rescue and screened for GFP fluorescence. Five out of 18 showed detectable GFP signal. Pattern of GFP fluorescence was identical in all GFP positive lines.

4.4. *Live Imaging*

Dissected embryos were mounted on agarose pads. GFP and differential interference contrast (DIC) recordings were acquired at 10–20 s intervals (exposure time 100 ms, 2 × 2 binning) with a wide-field microscope (Zeiss Axioplan II) equipped with a digital camera (Hamamatsu Orca ER 12 bit). Image processing was done with Meta View Software (Universal Imaging Corporation).

4.5. *Staining of single embryos*

Wild-type embryos were dissected in M9 buffer containing Hoechst 33342 10 mg/ml (Molecular Probes, Eugene, Oregon, United States) and FM 4-64 32 mM (Molecular Probes, Eugene, Oregon, United States). Embryos were washed briefly with M9 medium, and mounted on agarose pads.

Chitinase-treatment was performed as described in (Wolf et al., 1983). After treatment the embryos were placed in minimal EGM to avoid osmotic shock (Edgar, 1995).

4.6. *Electron microscopy*

Either whole worms or individual embryos collected into capillary tubing were cryoimmobilized using an EM PACT2 + RTS (Leica Microsystems) high-pressure freezer (Manninen et al., 2005; Muller-Reichert et al., 2003). Samples were freeze substituted at −90 °C for 2 days in acetone containing 1% osmium tetroxide and 0.1% uranyl acetate (Muller-Reichert et al., 2003). The temperature was raised progressively to room temperature over 22 h in an automatic freeze-substitution machine (Leica EM AFS). Samples were embedded in Epon/Araldite and thin sections (70 nm) were cut using a Leica Ultracut UCT microtome. Sections were collected on Formvar-coated copper grids, poststained with 2% uranyl acetate in 70% methanol followed by aqueous lead citrate and viewed in a TECNAI 12 (FEI) transmission electron microscope.

4.7. *Preparation and fractionation of a lipidic extract and bioassay*

Worms of *daf-22(m130)*, *emb-8(hc69)* and *zyg-1(b1)* strains were synchronized by putting eggs on plates without food. After 12 h, the starved L1 population was moved to ten 14.5 cm plates with bacteria. *emb-8(hc69)* was directly shifted to 25 °C degrees whereas *zyg-1(b1)* were moved at the L3 stage. After three days, gravid worms were collected by rinsing with ice-cold water, transferred into 50 ml Falcon tubes and pelleted (2 min at 1000 rpm). After the bleaching procedure, the collected embryos were subjected to three cycles of freezing–thawing (liquid N_2/sonication bath). The suspension was transferred into a glass bottle with 19 volumes of methanol containing 10 μg/ml of the antioxidant BHT. The extraction was performed overnight at room temperature with continuous agitation. Extracts were separated from worm remnants by filtration through a Whatman GF/A glass filter. Methanol extracts were combined and extracted two times with an equal volume of hexane. This methanolic extract was designated Fraction A, the hexane – as Fraction B. The two fractions were dried under N_2 flow, and dissolved in an appropriate volume of methanol or hexane (100 μl solvent per 1.10^6 eggs).

Testing the biological activity of lipidic fractions was performed in 12-well cell culture plates (Nunc, Roskilde, Denmark). Each well contained 1 ml of NGM agar mixed with 0.1% tergitol. Twenty microliters of Fractions A and B, or corresponding solvents, were added per well and dried in a laminar flow cabinet. Because we noticed that methanol had a significant toxicity, we first dried a portion of Fraction A in a speed vac, re-suspended it in isopropanol and then used it in the bioassay. Before seeding worms, 50 μl of bacteria for RNAi by feeding (prepared as described above) were added to plates and left overnight at room temperature. Around 30 *rrf-3(pk1426)* eggs/well were grown for one generation and after 100 h seven gravid adults were transferred to a new plate to lay eggs for two hours. Twenty-four hours after removing the mothers, L1 and non-hatched eggs were counted and embryonic lethality was assessed by determining the percentage of hatched L1 to the total eggs laid. Duplicates of each fraction per experiment were analyzed.

Acknowledgments

We are indebted to Tony Hyman (MPI-CBG) and members of his lab for providing several lines of worms and the initial help with microscopic studies. We are extremely grateful to

Carrie Cowan (IMP, Vienna) for critical reading of the manuscript and many helpful suggestions. We thank members of the Kurzchalia-lab for permanent interest and discussions. We thank Judith Nicholls for proofreading the manuscript. We want to acknowledge CGC for providing worm strains. We want to thank S. Mitani and the National Bioresource Project for the Nematode (Japan) for isolating and providing *tm2711* and *tm3224*. This project was partially supported by SFB/TR 13 grant from Deutsche Forschungsgemeinschaft to TVK (project B2) and Human Frontier Scientific Program.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mod.2009.02.001.

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