Regulation of the maternal-to-embryonic transition in *C. elegans*

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Susanne Finger
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Dr. Francesca Palladino
Dr. Antoine H. F. M. Peters
Dr. Rafal Ciosk

Basel, den 22. Mai 2012

Prof. Dr. Martin Spiess
(Dekan der Philosophisch-Naturwissenschaftlichen Fakultät der Universität Basel)

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Summary

Reproduction is a fundamental process of life which allows the survival of each species through the birth of young species members. Several modes of reproduction evolved and are classified into sexual and asexual reproduction. Sexual reproduction involves the fusion of two gametes: the female oocyte and the male sperm. Parthenogenesis is a form of asexual reproduction in which oocytes alone develop into viable progeny. Parthenogenesis most likely independently evolved multiple times from sexual reproduction and has been described in various invertebrate and vertebrate species.

In certain phyla, like in nematodes, closely related species differ in their modes of reproduction. Studying reproduction and early embryogenesis in these species allows the identification of molecular mechanisms which permit or restrict parthenogenesis. From other model organisms, several regulatory candidates are known. For example in flies, sperm independent oocyte activation and spindle formation facilitate parthenogenetic development, whereas in mammals, genetic imprinting, sperm dependent oocyte activation and oocytic centriole elimination restrict parthenogenesis.

We study the sexually reproducing nematode *C. elegans* in which parthenogenetic development has not been described. We analyzed the influence of maternally inherited epigenetic modifiers and found that modifiers involved in maintaining the soma - germ line distinction have no additional role in regulating the onset of embryonic transcription. We further analyzed embryonic features in two different maternal mutant backgrounds. We found that formation of a maternal germ line tumor, the teratoma, has striking similarities with early embryogenesis. In contrast, ovulated but unfertilized oocytes of a feminized mutant show early embryonic-like features but are unable to differentiate. The ability to differentiate is most likely not due to sperm inherited components but partially depends on the sperm triggered formation of a functional eggshell.

We conclude that in *C. elegans*, the onset of the maternal-to-embryonic transition is uncoupled from sperm dependent oocyte activation which might facilitate parthenogenesis in nematodes. Further, next to sperm dependent oocyte activation and centriole inheritance, sperm licensed eggshell formation might restrict parthenogenetic development in *C. elegans*.

1. Introduction

1.1. Beginning of new life: modes of reproduction

Every organism originates from other organisms of the same species through a process called reproduction. Thus, reproduction as a fundamental feature of life allows the birth of young species members and by this ensures the continuity and survival of each species. Several modes of reproduction evolved which are classified into sexual and asexual reproduction. Both ways of reproduction share common concepts but differ in other key events which will be described in the following chapters.

1.1.1. Sexual reproduction

In sexually reproducing species, new life develops after the fusion of two gametes - the female oocyte and the male sperm. Due to meiotic cell division, both gametes carry a haploid genome which they both pass on to the offspring. Thus, each progeny starts off with a diploid genome which is genetically different from each parent. Both, oocyte and sperm, are highly differentiated, yet together they give rise to a totipotent founder cell, called zygote, which initiates embryogenesis and is able to develop into a complex multicellular organism. This zygote initially has to overcome the germ cell character of its parental origin and create an environment in which it can control its further embryonic development (see chapter 1.2.). As the early embryo is transcriptionally silent, this process depends on maternally and paternally provided factors.

1.1.1.1. Maternal contribution

Both sperm and oocyte contribute equally to the zygotic genome but the cytoplasm is almost exclusively maternally inherited. This fact already becomes apparent by the size difference of the oocyte and sperm (Figure 1). The oocyte with its enormous cytoplasm outcompetes sperm, in which the cytoplasm is reduced to a minimum, by size. After fertilization, the zygotic cytoplasm is therefore largely identical with the maternal cytoplasm. The cytoplasm of a mature oocyte contains next to nutrients and mitochondria a

set of proteins and mRNAs which is stored in the oocyte for the purpose of embryonic development.

Among maternally contributed proteins are ribosomes and other proteins which ensure proper translation, but also several components of the polymerase II pre-initiation complex (PIC). Studies in the fly *Drosophila melanogaster* (*D. melanogaster*) showed that components of the general transcription factor complex TFIIH are deposited maternally into the cytoplasm (Aguilar-Fuentes et al., 2006). Functional polymerase II (Pol II) proteins are maternally inherited in the mouse *Mus musculus* (*M. musculus*) and the frog *Xenopus laevis* (*X. laevis*) and used by the early embryo at a specific time point during embryonic development at which transcription is reinitiated (Latham et al., 1992, Roeder, 1974). Next to PIC components, studies in mouse and the nematode *Caenorhabditis elegans* (*C. elegans*) detected chromatin remodeling factors which are loaded into the oocyte but function in the early embryo. For example, the mouse SWI-SNF (SWItch/Sucrose NonFermentable) component BRG-1 is present in oocytes and embryos exhibit an early developmental arrest if BRG-1 is maternally depleted (Bultman et al., 2006). In *C. elegans*, the H3K36 methyltransferase MES-4 is present in oocytes and acts in embryos where it is important for proper germ line development in the offspring (Capowski et al., 1991, Bender et al., 2006, Fong et al., 2002). But also early lineage specification proteins are loaded into the oocytic cytoplasm, as in the case of *C. elegans* PIE-1 which is essential for germ cell identity in early germ line blastomeres (Mello et al., 1996).
Notably, although these proteins are physically present in a functional form, they are most often subject to general regulation. For example, most of the known maternally provided proteins which are directly involved in active transcription and thus function in the nucleus are kept in the cytoplasm and only translocate into the nucleus at a specific time point during embryonic development (Guven-Ozkan et al., 2008, Aguilar-Fuentes et al., 2006, Torres-Padilla and Zernicka-Goetz, 2006).

Maternally provided mRNAs are another essential group of inherited developmental regulators. They and their protein products are important for embryonic body axis specification and pattern formation and ensure correct timing of embryonic transcription, as has been proposed for mouse CycA2 (Hara et al., 2005). In *D. melanogaster*, the maternal mRNAs *bicoid* and *nanos* are localized to opposite poles in the oocyte and by this determine the anterior-posterior axis of the developing embryo (Gavis and Lehmann, 1992, Lehmann and Nusslein-Volhard, 1991). In *X. laevis* oocytes, the mRNAs Vg1 and VegT are enriched in the vegetal pole and determine the zygotic dorsal-ventral axis (Kataoka et al., 2005).

Another group of maternally inherited mRNAs encode for transcription factors which drive lineage specification in the developing embryo as has been extensively studied in *C. elegans*. For example, the *C. elegans* transcription factor Caudal/PAL-1 is maternally inherited and largely responsible for embryonic muscle development by activating the muscle specification factor HLH-1 (Hunter and Kenyon, 1996, Fukushige and Krause, 2005).

Generally, the translation of maternally provided mRNAs is highly regulated and usually repressed during oogenesis. Translational repression in oocytes is mostly achieved by regulating the poly(A) tail length of mRNAs in *X. laevis* and *M. musculus* and at least partially also in *D. melanogaster* and *C. elegans* (Hodgman et al., 2001, Kim and Richter, 2006, Salles et al., 1994, Kim et al., 2010). A short poly(A) tail usually indicates transcriptional repression whereas polyadenylated mRNAs stimulate their own translation. As deadenylated mRNAs are prone for 3' to 5' degradation, additional regulators have to ensure stabilization of these mRNAs. Several studies suggest that RNA stabilization is achieved by repressing miRNA function in mouse, inhibiting the 3' to 5' decay pathway in *X. laevis*, and binding of specific RNA binding proteins to their target transcripts in *D. melanogaster* and *C. elegans* (Ma et al., 2010, Suh et al., 2010, Voeltz and Steitz, 1998, Mancebo et al., 2001, Scheckel et al., 2012). Although maternally provided mRNAs are repressed during oogenesis, these mRNAs can be rapidly translated into functional proteins in the early embryo. Therefore, cytoplasmic storage of mRNAs is a

potent way to provide embryos with essential developmental factors which otherwise would interfere with proper oogenesis.

Taken together, the oocyte with all its stored nutrients, proteins and mRNAs provides the foundation for embryogenesis but especially in obligatory sexually reproducing species, sperm derived components are essential for initiating and/or progressing through embryogenesis.

1.1.1.2. Paternal contribution

Whereas the capacious oocytic cytoplasm is loaded with nutritious and regulative components, the sperm cytoplasm is reduced to a minimal size, carrying only a limited number of organelles and factors.

Sperm organelles include mitochondria which are important during spermatogenesis and provide energy for sperm motility. Although sperm mitochondria enter the oocyte after fertilization, only the maternally derived

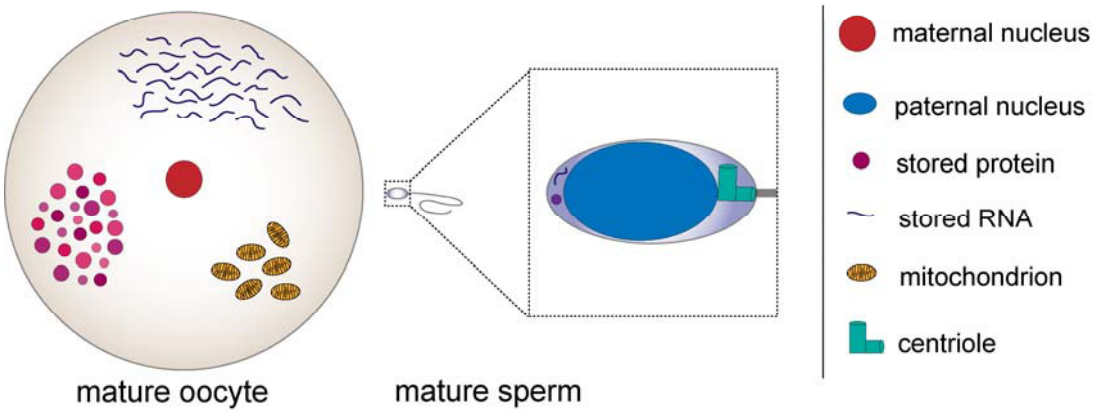


Figure 1: Oocyte versus sperm - differences in size and inheritable components
Mature human oocytes reach approximately 150 µm in diameter whereas the heads of mature human spermatozoa reach approximately 3.5 x 7 µm (height x width). Both, oocyte and sperm, contribute equally to the genome of the offspring. Yet, the mature oocyte carries a variety of RNAs and proteins which are inherit to the zygote and is the sole donor of mitochondria. Mature sperm also inherits RNAs and proteins to the zygote, although their numbers are much lower and their significance not well understood. Further, sperm is the sole doner of centrioles in most species.

mitochondria are truly inherited by the embryo. Paternally derived mitochondria are instead actively degraded through macroautophagy in the early embryo and are not detectable anymore after the eight cell stage in *M. musculus* and the 16 cell stage in *C. elegans* (Sato and Sato, 2011, Cummins et al., 1997).

Studies identified low levels of mRNAs in mature mammalian sperm (Pessot et al., 1989, Yang et al., 2009, Lalancette et al., 2009). Amongst the identified transcripts in humans are mRNAs encoding for transcription factors, cell cycle components and sperm specific protamines which enable tight DNA packaging (Siffroi and Dadoune, 2001, Dadoune et al., 2005, Wykes et al., 1997). Functional gene group analysis revealed that the most abundant transcripts cluster into groups of nuclear proteins involved in transcription, and cytoplasmic proteins involved in protein translation (Zhao et al., 2006). The majority of these mRNAs is not stored for embryonic development but for late stages of spermatogenesis in which transcription has been shut down (reviewed in Steger, 1999). Nevertheless, sperm derived mRNAs are known to be delivered to the oocyte at fertilization (Ostermeier et al., 2004). Further, sperm derived mRNA is able to influence the offspring phenotype as has been shown in *Kit* +/+ homozygous mice received from *Kit* +/- heterozygous males. These males transmit the white-spotted *Kit* phenotype to their progenies by an aberrant *Kit* spermatozoal mRNA (Rassoulzadegan et al., 2006). However, the role of other sperm mRNAs and their potential function during early embryonic development remains elusive.

So far, little is known about paternally inherited proteins which are essential for embryogenesis. Proteomic analyses of mature sperm mainly identified proteins which are important during spermatogenesis or influence sperm motility and oocyte-sperm interaction (Baker et al., 2007). Studies in *C. elegans*, identified the protein SPE-11 to be inherited by sperm but being functional only in the zygote where it is important for establishing the polyspermy barrier and generating a functional eggshell (Johnston et al.,

2010, Browning and Strome, 1996). However, SPE-11 has no known homolog outside the nematode phylum.

Centrioles are essential sperm derived factors which ensure proper embryogenesis. In most sexually reproducing species it is only the sperm which carries and inherits a pair of centrioles into the developing zygote. Centrioles are essential for generating functional centrosomes which serve as microtubule organization centers (MTOC) and enable mitotic spindle formation and subsequent progression through mitosis (reviewed in Schatten, 1994). In addition to their general function as a MTOC, centrosomes temporally localize mitotic regulators and ensure accurate entry into mitosis. For example, they concentrate the Aurora A kinase at its site of action which is important during the first zygotic cell division when the paternal and maternal pronuclei synchronously undergo nuclear envelope breakdowns and fuse (Hachet et al., 2007). Moreover, in *C. elegans,* sperm donated centriole support the generation of anterior-posterior polarity in the zygote which is essential for proper embryogenesis (Zonies et al., 2010). Notably, not all species inherit centrioles through sperm. For example, rodent sperm does not contain centrioles and centrosomes are assembled de novo in zygotes by maternally provided components (Schatten et al., 1986). For a detailed description of centrosome reduction during gametogenesis, see chapter 1.1.2.3..

Another important function of sperm is its involvement in oocyte activation. Mature oocytes are poised to begin embryogenesis but arrest in a state in which meiosis is not completed and the outer oocyte layer is specialized to allow sperm binding. Immediately after fertilization, intracellular calcium levels rise, beginning at the sperm entry point and further spread across the zygote (Gilkey et al., 1978, Eisen et al., 1984). The increase of calcium levels triggers a variety of biochemical cascades collectively known as oocyte activation. Two main processes affected by the change in calcium levels are the release of the meiotic arrest and changes in the outer layer to create a polyspermy barrier (Tatone et al., 1999, Liu and Maller, 2005). In mammals, the initial

calcium increase is triggered by the sperm-specific phospholipase C zeta which is introduced into the oocyte at fertilization (Saunders et al., 2002).

Taken together, there are much less paternally inherited factors which are essential for the early embryonic development compared to maternally derived components (Figure 1). Yet the few known factors are of equal importance and ensure the development into viable offspring.

1.1.2. Parthenogenesis as a form of asexual reproduction

In asexually reproducing species, progeny arise as a genetically identical or similar copy of its single parent. For example, bacteria and yeast are able to "split" themselves into one or more identical progeny. Fragmentation occurs in animals like corals and hydras when organisms split into several fragments from which each is able to develop into a mature "clone" of the original organism. Naturally, these kinds of asexual reproduction do not involve or depend on gametogenesis.

Another form of asexual reproduction is parthenogenesis - Greek for "virgin birth". Parthenogenesis defines the embryonic development of unfertilized oocytes leading to viable, usually female, progeny which are genetically similar to their mother. Parthenogenesis occurs naturally in a variety of invertebrates, e.g. aphids and nematodes, but also in vertebrates, e.g. lizards and sharks. Parthenogenetically reproducing species are often also able to reproduce sexually and exhibit a cyclic or male-occurrence depending switch of reproductive modes, e.g. aphids and sharks, respectively. As oocytes are highly differentiated cells themselves, these species have to overcome the sperm dependence during initiation of embryogenesis. Each species thus developed a variety of mechanisms for oocyte activation, ploidy maintenance in progenies, and centrosome assembly in the zygote.

1.1.2.1. Oocyte activation in parthenotes

Most species rely on sperm entry as a trigger for oocyte activation (see chapter 1.1.1.2.). However, in several insect species oocyte activation is

initiated by mechanical forces during ovulation. *D. melanogaster* oocytes, for example, are squeezed out of the ovary into a narrow oviduct where, additionally, they take in fluid and swell. *D. melanogaster* oocytes can be *in vitro* activated by incubation in hypotonic media (Page and Orr-Weaver, 1997). The rate of artificial oocyte activation increases if in addition to the hypotonic media hydrostatic pressure is applied (Horner and Wolfner, 2008). These findings indicate that *in vivo* both stimuli, squeezing through a narrow oviduct and swelling through fluid uptake, lead to oocyte activation. More recently, a molecular pathway has been suggested in which calcium ions from the external fluid can enter the oocyte through stretch-activated ion channels and in addition to an internal calcium ion release might trigger oocyte activation (Horner and Wolfner, 2008, Adams et al., 1998). Indeed, in *Drosophila mercatorum*, a close relative of *D. melanogaster*, a low percentage of unfertilized oocytes spontaneously develop into viable female flies which indicates successful oocyte activation by similar mechanisms (Carson, 1967). Further, when oocytes dissected from the wasp *Pimpla turionellae* are squeezed through a narrow capillary, the majority develop into larvae (Went and Krause, 1974). Thus, mechanical and osmotic pressure successfully activate oocytes and these kinds of stimuli are adapted by parthenogenetically reproducing species.

1.1.2.2. Maintaining ploidy levels in parthenotes

Gametogenesis usually involves meiosis, or chromosome reduction, leading to haploid genomes. In sexually reproducing species, two haploid gametes fuse and give rise to a diploid progeny. In parthenogenetically reproducing species, no other partner contributes to the genome of the progeny, so that females have to provide their offspring with a non-reduced set of chromosomes. In most species, females will have to provide a diploid set of chromosomes. However, especially in obligatory parthenogenetically reproducing species, the ploidy level can be dramatically different. For example, the grasshopper *Saga pedo* has a pentaploid set of chromosomes and the false spider mite has a haploid genome (Dutrillaux et al., 2009, Weeks

et al., 2001). Generally, it is believed that a polyploid genome is of advantage to obligatory parthenogenetically reproducing species as it minimizes the risk of demasking recessive lethal mutations (reviewed in Archetti, 2010).

One possibility to maintain ploidy levels in parthenogenetically reproducing species is to completely suppress meiosis. In this case, primary oocytes divide mitotically and give rise to mature oocytes. This process of oocyte formation is referred to as apomixis and leads to the formation of genetically identical offspring (Figure 2). In animals, apomixes has been shown in certain aphid species and bdelloid rotifers (Mark Welch et al., 2003, Blackman and Spence, 1996).

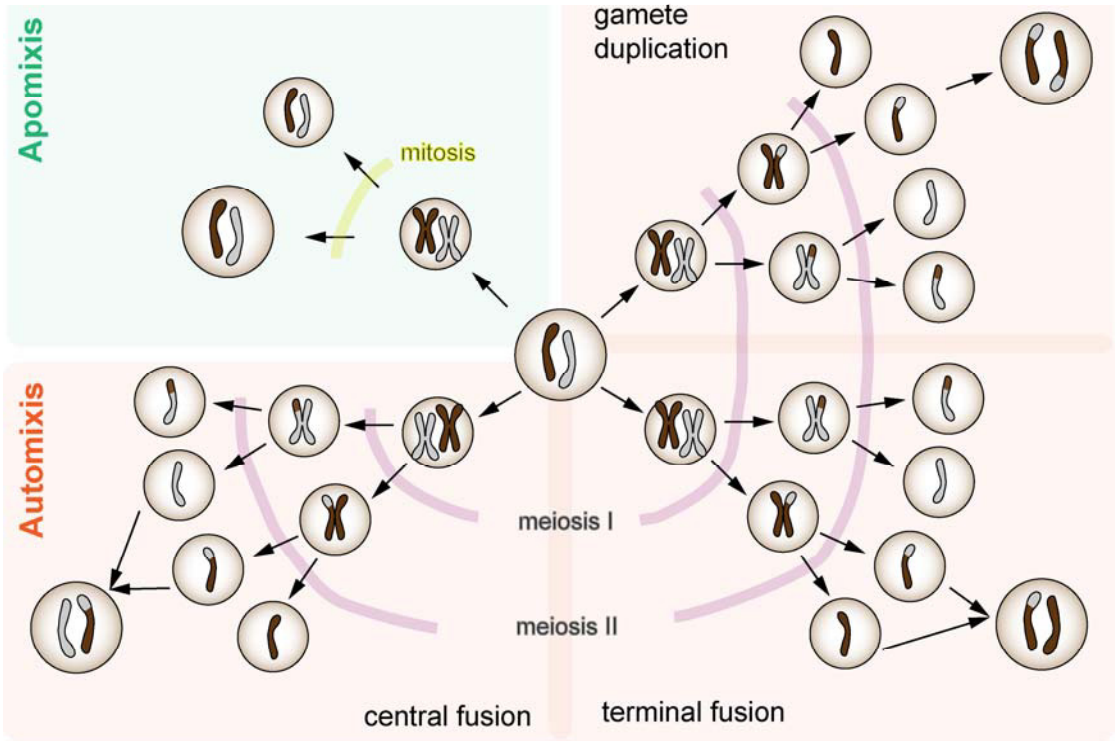


Figure 2: Apomixis and forms of automixis

Apomixis (top left) describes oocyte formation in which meiosis is blocked and progenies are identical to their mother. On the other hand, automixis describes oocyte formation in which meiosis, including recombination, takes place and progenies are genetically variable. In the case of gamete duplication (top right), oocytes duplicate their genome. Terminal fusion (bottom right) involves the fusion of sister nuclei, whereas central fusion (bottom left) involves the fusion of non-sister nuclei.

Automixis is a second and more complex form to maintain chromosome ploidy (Figure 2). In automictic oocyte development, meiosis takes place but ploidy levels are restored during or after meiotic cell divisions. For example, duplication of a haploid set of chromosomes during the first mitotic cell cycle after meiosis will lead to a diploid genome. This kind of “gamete duplication” has been shown in the wasp *Leptopilina clavipes* (Pannebakker et al., 2004). Further, before or after meiosis II, haploid daughter cells are able to fuse. If the fusion occurs between sister nuclei, this kind of automixis is called “terminal fusion”. A fusion between non-sister nuclei is referred to as “central fusion”. As automixis is a specialized kind of meiosis, recombination happens normally und progenies are genetically variable.
Automixis has been described in several insect species and is further believed to cause facultative parthenogenesis in vertebrates (reviewed in Mogie, 1986, Chapman et al., 2007). Molecular mechanisms underlying apo- and automictic development are unfortunately not known.

1.1.2.3. Centrosome formation in parthenotes

Centrosomes consist of a pair of centrioles and a surrounding pericentriolar matrix. Each cylindric centriole is usually made up of nine symmetrically oriented microtubular triplets. The two centrioles forming a pair are orthogonally oriented and consist of an older “mother” centriole and a younger “daughter” centriole which can be discriminated by several appendages. The fibrous pericentriolar matrix surrounds the mother centriole and consists of a variety of proteins which establish centrosome anchoring and microtubule nucleation. During gametogenesis of sexually reproducing species, centrosomes become inactive and are partially or completely degraded. The kind of centrosome reduction differs between spermatogenesis and oogenesis but centrosomes are reestablished after oocyte-sperm fusion.

Centrosome reduction during spermatogenesis includes partial or complete degradation of pericentriolar proteins and partial or complete degeneration of centrioles. For example, mouse and rat spermatozoa completely lose the

pericentriolar matrix and both centrioles (Woolley and Fawcett, 1973, Manandhar et al., 1998, Manandhar et al., 1999). Non-rodent mammalian as well as invertebrate spermatozoa retain a small portion of pericentriolar proteins and the proximal daughter centriole, whereas the second distal centriole is degraded to various degrees (Manandhar and Schatten, 2000, Sathananthan et al., 1991, Manandhar et al., 2000, Dallai and Afzelius, 1991).

Centrosome reduction during oogenesis mainly involves the degeneration of centrioles during early or late stages of meiosis. For example, in mammals, centrioles are present until the pachytene stage of meiosis I but are absent in subsequent meiotic stages (Szollosi et al., 1972). Consequently, meiotic spindle formation takes place in the absence of centrioles (Hertig and Adams, 1967, Szollosi et al., 1972). From mouse studies it is known that multiple MTOCs appear before metaphase I which gradually combine to form the metaphase spindle (Calarco et al., 1972). On the other hand, in the snail *Lymnaea stagnalis*, centrioles are present in mature oocytes but do not duplicate before meiotic division I. Thus, spindle poles of meiosis I contain only one centriole which is further distributed to the meiosis II outer spindle pole and extruded with the second polar body (Krioutchkova et al., 2002). In contrast to centriole elimination, starfish oocytes retain one centriole which is present but not functional in the early embryo (Sluder et al., 1989).

In summary, centrosome reduction during gametogenesis is a reciprocal event: During spermatogenesis, pericentriolar proteins are degenerated, whereas oogenesis includes the loss of centrioles. Only after fertilization, paternally inherited centrioles combine with maternally inherited pericentriolar matrix proteins to give rise to a functional centrosome. Despite intensive research, the precise molecular mechanisms underlying centrosome reduction are poorly understood. However, work from *C. elegans* indicates that the karyotype as well as somatic cells contribute to centriole elimination during oogenesis and that centriole elimination is delayed in the absence of the RNA helicase CGH-1 (Mikeladze-Dvali et al., 2012).

As centrosomes are the major MTOC in cells, parthenogenetically reproducing species have to adapt mechanisms to form centrosomes in the absence of sperm-inherited centrioles. Theoretically, it should be possible to inhibit centrosome reduction during oogenesis and thus maintain functional centrosomes in mature oocytes. This would be of great advantage to species as centrosome replication based on a pre-existing template is thought to be more efficient than *de novo* synthesis (reviewed in Marshall, 2001). However, there is no parthenogenetically reproducing species known in which centrosome reduction is inhibited (reviewed in Engelstadter, 2008). Parthenogenetically reproducing animals therefore rely on *de novo* synthesis of centrioles and developed mechanisms to ensure synthesis of the proper numbers of centrosomes. Studies in the wasp *Nasonia vitripennis* detected multiple cytoplasmic asters in the post-meiotic oocyte (Tram and Sullivan, 2000). These asters contain centrosomal proteins as well as centrioles and behave like centrosomes in the way they replicate and split (Riparbelli and Callaini, 2003). If the oocyte is fertilized, the asters disappear. In the absence of sperm, however, two of these asters stably associate with the pronucleus and develop into centrosomes (Tram and Sullivan, 2000). Similar mechanisms have been described in the aphid *Acyrthosiphon pisum*, the wasp *Muscidifurax uniraptor*, and the fly *Drosophila mercatorum* (Riparbelli et al., 1998, Riparbelli et al., 2005, Riparbelli and Callaini, 2003). Interestingly, aster formation in *Drosophila mercatorum* occurs in 65 % of unfertilized oocytes, yet only 8-10 % of unfertilized oocytes develop into viable progeny. As various numbers of *de novo* synthesized centrosomes can be detected in unfertilized oocytes, it is interesting to speculate whether the low survival rate of unfertilized oocytes is due to an insufficient inactivation of abnormal centrosome numbers (Riparbelli and Callaini, 2003).

Taken together, parthenogenetically reproducing species synthesize centrosomes de novo by spontaneously assembling centrosomal components and regulating the formation of precise numbers of centrosomes. How this is achieved on molecular basis is, however, not yet understood.

1.1.3. Advantages and disadvantages of sexual reproduction versus parthenogenesis

Both, sexual reproduction and parthenogenesis are evolutionary well established modes of reproduction with each mode having certain short- and long-term advantages and disadvantages.

Parthenogenetically reproducing females are theoretically able to produce twice as many daughters as sexual females. These daughters are able to reproduce from the moment they develop oocytes. Sexual females which reach the reproductive age have to invest time and energy to find a mating partner and risk to be harmed through the mating process. Starting with a small population, parthenogenetically reproducing species will therefore increase their population size faster than sexually reproducing species. Offspring of parthenogenetically reproducing females inherit their genome 100 % from their mother, whereas sexually reproducing females “dilute” their own genetic contributions by 50 %. Parthenogenetically reproducing species therefore lower the risk of losing highly successful gene combinations, whereas sexually reproducing species accept the possibility of creating less favorable gene combinations. Mathematically, these advantages of parthenogenesis over sexual production have been described in the “two-fold cost of sex” theory (reviewed in Maynard Smith, 1978).

But if parthenogenesis is favored over sexual reproduction, why are sexually reproducing species more abundant? And why do most of the parthenogenetically reproducing species switch to sexual reproduction within their seasonal population cycle or as soon as males are present?
It is widely accepted that parthenogenetically reproducing species lose their genetic variability over time. For example, every individual of an apomicticly reproducing population is genetically identical - with the exception of random mutations. And even automicticly reproducing parthenotes, in which meiotic recombination takes place, will acquire a highly homozygous genome after a few generations. The general loss of heterozygosity leads to demasking of detrimental alleles and thus has a harmful influence on offspring fitness, as it

is known for inbreeding populations (reviewed in Charlesworth and Charlesworth, 1987). Sexually reproducing species avoid the risk of inbreeding depression by recombining different sets of genes in every single progeny. By doing so, species accept the possibility that a couple of offspring inherit unfavorable gene combination but in this case fitness levels are only reduced in a minor part of the population. On the other hand, in every population there will be individuals which inherit favorable gene combinations resulting in a high population fitness level. The extent of genomic variation in sexually reproducing species is believed to have several advantages as it allows fast adaptation in host-parasite interactions and efficiently eliminates harmful mutations (reviewed in Kondrashov, 1988, Salathe et al., 2008)

Taken together, parthenogenetically reproducing species increase their population size quickly but at the same time lose their heterozygosity which leads to overall poor adaptation skills. Such a population therefore has a high short-term advantage but is over time more vulnerable to changes in their environment. On the other hand, sexually reproducing species are slower in expanding but acquire an enormous genetic variability in their population which is of huge advantage in the long-term survival of species.

1.1.4. Parthenogenetic development in strictly sexually reproducing species

Parthenogenesis and sexually reproduction are not mutually exclusive. Most species in which parthenogenesis has been described are also able to reproduce sexually if males are present. Even oocytes from species which have been described as exclusively sexually reproducing, can be activated sperm independently. In the absence of sperm, they mimic very early embryonic development although they will never produce viable offspring. In research, these activated oocytes are often also referred to as “parthenotes”. To avoid confusion between viable parthenotes derived from true parthenogenesis and inviable “parthenotes” derived from strictly sexually reproducing species, I will mark the latter with quotation marks (“…”).

1.1.4.1. Non-mammalian “parthenogenetic” development

In *D. melanogaster*, oocyte activation occurs before fertilization by sperm (see chapter 1.1.2.1.). Analysis of oocytes collected from virgin fly females revealed that unfertilized fly oocytes finish meiosis (Doane, 1960). Activated oocytes further form a functional eggshell as eggshell formation naturally occurs in the oviduct whereas fertilization takes place through a micropyle in the fly uterus (Heifetz et al., 2001). Several maternal mRNAs are translated leading to an increase of protein levels similar to those observed for fertilized embryos. Further, degradation of a subset of maternal mRNAs occurs similar to fertilized embryos (Bashirullah et al., 1999). Activated but unfertilized *D. melanogaster* oocytes are, however, unable to reinitiate transcription and therefore do not progress through the maternal-to-embryonic transition (see chapter 1.2.).

X. laevis oocytes can be artificially activated by pricking with a needle. These activated oocytes show “parthenogenetic” development at very low frequency of approximately 1 % (Tournier et al., 1989). Most of the activated oocytes exhibit surface contraction waves similar to control fertilized oocytes (Hara et al., 1980). However, most of the activated but unfertilized oocytes are not able to initiate the first mitotic cleavage unless centrioles are experimentally injected. In this context, even centrioles extracted from human cells lead to “parthenogenesis” in frog oocytes until the blastula stage (Tournier et al., 1989).

No “parthenogenetic” development has been described in the nematode *C. elegans*. Yet, ovulated but unfertilized oocytes are known to progress through an abnormal meiosis in which anaphase I is completed but no meiosis II spindle forms (McNally and McNally, 2005). Further, these oocytes endoreplicate but no cell cleavages take place (Ward and Carrel, 1979).

“Parthenogenetic” development initiates from oocytes in the absence of sperm. If “parthenogenetically” activated oocytes are not yet ovulated, a germ line tumor - the teratoma - can arise (see chapter 1.1.4.2.). A teratoma is a

usually benign tumor which consists of differentiated tissue of all three germ layers and occurs in both female and male germ lines. In the *C. elegans* teratoma model, teratoma formation occurs in the adult and thus female germ line if the RNA binding protein GLD-1 is depleted (see chapter 1.3.1.1. for details) (Ciosk et al., 2006). Within the *gld-1* dependent teratoma, cells differentiate into muscles, neurons and at low frequency also into intestinal cells (Ciosk et al., 2006). Further, early embryonic genes which are usually expressed at the point of embryonic gene activation in wild type embryos are also detected in the teratoma, indicating that teratoma formation indeed mimics embryonic development (Biedermann et al., 2009).

1.1.4.2. Mammalian “parthenogenetic” development

Mammals are unique amongst the animal kingdom in the way that no mammalian species is capable of true parthenogenetic reproduction. However, spontaneously or artificially activated mammalian oocytes undergo “parthenogenetic” development until a species-specific time point during embryogenesis. For example, mice “parthenotes” are able to develop up to the forelimb bud stage and also rabbit “parthenotes” develop up to day 10-11 of embryogenesis despite showing growth defects (Kaufman et al., 1977, Ozil, 1990). “Parthenotes” derived from primates, however, fail to develop beyond the implantation stage (Marshall et al., 1998).

Oocyte activation in mammals normally requires fertilization. However, spontaneous oocyte activation can occur and, if the oocyte still resides in the ovary, is thought to be the main reason for ovarian teratoma formation. Studies in Mos-deficient female mice support this theory. Mos -/- females frequently develop ovarian teratomas and a fraction of *in vitro* matured oocytes of this mouse strain “parthenogenetically” develops into the blastocyst stage. Further, pre- and early post-implantation embryos were discovered in ovaries of Mos -/- females (Hirao and Eppig, 1997). A case report in a human female patient also links “parthenogenetic” development to teratoma formation. The patient was diagnosed with an ovarian teratoma but underwent

in vitro fertilization (IVF) later during her life. Ovarian stimulation during the IVF procedure resulted in the recovery of “parthenogenetically” developing oocytes (Oliveira et al., 2004).

Spontaneous oocyte activation can also occur after ovulation. Under certain circumstances, these activated oocytes can be fertilized leading to the development of “parthenogenetic” chimaera. “Parthenogenetic” chimaera which were artificially produced in mice by fusing cells of very early “parthenotes” and fertilized embryos are viable (Stevens et al., 1977). However, cells of “parthenogenetic” origin are systematically eliminated in most of the tissues like muscle, liver and pancreas whereas a minor contribution could be found for brain, heart, kidney and spleen tissues (Fundele et al., 1989, Nagy et al., 1989). Studies in mice in which “parthenogenetically” activated oocytes were fertilized indicate that fertilization during the first mitotic cell cycle of the oocyte leads to the incorporation of paternal chromosomes during the second cell cycle. However, during the first cell division, the sperm pronucleus is passively segregated to only one cell of the two cell stage “parthenogenetic” chimaera, so that only one cell inherits contributions from both parents whereas the second cell inherits only maternal components (Maleszewski, 1992). In humans, at least two case studies have been reported in which spontaneous oocyte activation followed by fertilization is the most likely explanation for the observed genotypes of the “parthenogenetic” chimaeric patients. In one case, a phenotypical male patient had a female blood karyotype which could be identified as being of only maternal origin. Skin fibroblasts of this patient, on the other hand, were positive for allelic markers of both parents with identical maternal alleles as the female blood cells (Strain et al., 1995). The second study describes a more complex genotype in a patient who most likely arose from double fertilization of a “parthenogenetically” activated oocyte. This patient appears to be male but was diagnosed with hermaphroditism as his internal reproductive organs contained both testicular and ovarian tissues. The karyotype of the patient’s lymphocyte showed a mixture of cells with XY and XX chromosomes. Molecular analysis of the patient’s lymphocytes further identified the presence

of three alleles for several autosomal markers of which two alleles originated from the father (Giltay et al., 1998).

These case studies indirectly confirm the occurrence of spontaneous oocyte activation inside and outside the ovary. They also demonstrate the importance of biparental contributions to the offspring as “parthenotes” alone are not viable. The molecular mechanism underlying the need for biparental genomic contributions in mammals is known as genomic imprinting.

Genomic imprinting is a form of epigenetic inheritance in which gene expression is restricted to only one allele, coming either from the mother or the father (Reik et al., 1987). Thus, genomic imprinting is already accomplished during gametogenesis when genes are differentially marked depending on whether they are going through oogenesis or spermatogenesis. The differential marking of genes is achieved by DNA methylation which is a reversible addition of a methyl group on the cytosine pyrimidine ring mainly at CpG dinucleotides (Li et al., 1993). CpG dinucleotides are often clustered into so called CpG islands within gene promoters and DNA methylation of these CpG islands correlates with gene silencing (reviewed in Bird, 2002). During genomic imprinting, DNA methylation occurs at CpG rich imprinting control regions (ICRs). Depending on their methylation status, ICRs either repress or enhance the expression of neighboring genes. So far, approximately 80 genes have been identified to undergo genomic imprinting (reviewed in Feil, 2009). The majority of these genes is methylated during late stages of oogenesis and is therefore only expressed from paternal alleles during embryogenesis. In mouse, only three genes are known to be methylated during spermatogenesis and thus allow maternal alleles to be expressed. Most of the protein coding genes regulate organogenesis, but also cell cycle regulators and non-coding RNAs, including miRNAs, are imprinted (Morison et al., 2005).

One key study demonstrated that genomic imprinting is indeed the barrier to parthenogenesis in mice. Therefore, nuclear transfer of naïve, non-imprinted, oocytes and fully grown, imprinted, oocytes was performed. Naïve oocytes

lacking the *H19* allele and the ICR of the *Igf2* allele mimic wild type genomic imprinting of these two alleles. A fraction of “parthenotes” derived from such a nuclear transfer were viable and developed into adulthood (Kono et al., 2004).

1.2. Maternal-to-embryonic transition

Oocyte activation - with or without sperm - it the first step of embryogenesis. The newly formed zygote is transcriptionally inactive and depends on parentally inherited regulatory elements like RNAs and proteins to initiate embryogenesis. As described earlier, the majority of RNAs and proteins are loaded into the oocyte and are therefore maternally provided, so that early developmental events in the embryo are under maternal control. At one point during early embryogenesis, maternally provided factors are exhausted and the embryo depends on newly synthesized mRNAs. Embryonic gene activation (EGA) is initiated at a species-specific time point during embryogenesis and first embryonically transcribed RNAs are detected. The embryo gradually takes over its own development until it becomes independent of maternal regulatory factors. The maternal-to-embryonic transition (MET) therefore describes the process of how the embryo acquires its independence.
In literature, the term MET (or MZT for maternal-to-zygotic transition) is used alternatively to either define the precise developmental time point at which the embryo has acquired its independence or the period from oocyte activation until the time point of independent embryonic control (reviewed in Tadros and Lipshitz, 2009, Baroux et al., 2008). I use it in the latter sense.

MET is an essential part of early embryonic development which differs between species. After oocyte activation through fertilization or other external stimuli, meiosis of the maternal nuclei continues and eventually finishes. Ploidy levels in the offspring are restored by either fusion of the male and female pronuclei in sexually reproducing species or by mechanisms described earlier in parthenogenetically reproducing species (see chapter 1.1.2.2). Mitotic divisions, either synchronous as in *D. melanogaster* or asynchronous as in *C. elegans*, restart and are followed by cytokinesis in most species. In many insect embryos, e.g. *D. melanogaster*, cytokinesis is blocked during the first cleavage cycles leaving the nuclei aligned to the cell membrane in a syncytium. Cytokinesis first reoccurs at the mid-blastula transition and shortly

afterwards gastrulation begins. Across species, gastrulation is a hallmark in embryogenesis and leads to the development of the three germ layers endo-, meso- and ectoderm, out of which differentiated tissues arise.

During these early embryonic steps, the maternal-to-embryonic transition takes place. MET comprises two intermingled processes, the degradation of a subset of maternally provided RNAs and proteins and the onset of embryonic transcription (Figure 3).

1.2.1. Maternal mRNA and protein degradation

A large proportion of maternally inherited mRNAs is degraded during early steps of embryogenesis. It has been estimated that at least 30 % of maternal mRNAs are degraded in *C. elegans* and similar numbers have also been reported for *D. melanogaster* (Baugh et al., 2003, De Renzis et al., 2007). For *M. musculus*, numbers vary from around 30 % to 80 %, but overall confirm a conserved massive maternal mRNA degradation (reviewed in Schultz, 2002, Hamatani et al., 2004).

Interestingly, studies in *D. melanogaster*, in which oocyte activation is independent from fertilization, identified a two step process of how maternal mRNA degradation is achieved. The first step is exclusively maternally

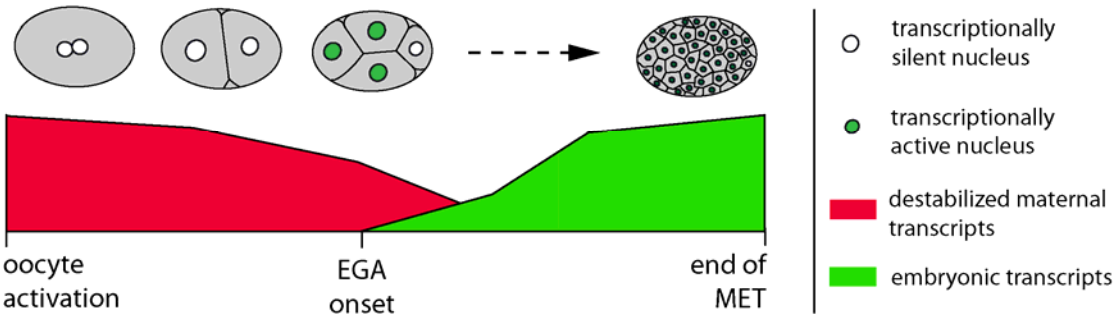


Figure 3: Schematic overview of the MET in *C. elegans*

Hallmarks of the MET are schematically depicted by expression profiles of destabilized maternal transcripts and embryonically transcribed RNAs (bottom). Correlating embryonic development is shown as an example for *C. elegans* embryogenesis (top).

regulated as it functions in the absence of embryonic transcripts, whereas the second step occurs only after embryonic transcription is initiated (Bashirullah et al., 1999). Gene expression profiling in mice and *C. elegans* further suggest that this two-step degradation activity is conserved among species (Baugh et al., 2003, Hamatani et al., 2004).

Studies in several model organisms identified two major classes of maternal mRNA destabilizing factors - RNA-binding proteins and small RNAs (Table 1). Both classes bind to motifs within the 3' UTR of their target mRNAs and recruit complexes which lead to deadenylation and destabilization of bound mRNAs.

For example, the fly RNA binding protein Smaug binds to motifs in the 3' UTR of its target mRNAs and recruits the CCR4/POP2/NOT-deadenylase complex (Tadros et al., 2007, Semotok et al., 2005). Deadenylation of maternal mRNAs leads to their degradation. *smaug* RNA itself is translationally repressed during oogenesis but after oocyte activation translational repression is released through the Pan gu Ser/Thr kinase complex (Tadros et al., 2007).
Several studies also suggest a role of Pumilio in maternal mRNA degradation in *D. melanogaster*. Pumilio-like binding motifs occur frequently in mRNAs which are enriched amongst embryonically degraded transcripts (Thomsen et al., 2010). So far, however, Pumilio has been shown only to directly degrade the *bicoid* mRNA in the fly embryo (Gamberi et al., 2002).

Work in *M. musculus* identified the zinc-finger RNA binding protein ZFP36L2 as deadenylation factor in early embryos (Ramos, 2012). Knockout females are infertile due to an early embryonic arrest at the two-cell stage, which in mice coincides with late stages of MET (Ramos et al., 2004). Although large scale destabilization of ZFP36L2 deadenylated mRNAs has not yet been investigated, these data suggest a potential role of ZFP36L2 in maternal mRNA degradation.
Deadenylation and degradation can be uncoupled, as shown in *X. laevis* embryos. The RNA binding protein EDEN-BP triggers deadenylation by

Table 1: Factors regulating maternal mRNA degradation during MET

organism	factor	factor type	maternal/embryonic step of degradation	known function	reference
C. elegans	MEX-5/6	zinc-finger protein	maternal	promotes degradation of *nos-2* mRNA in somatic blastomeres	D'Agostino et al., 2006
	miR-35-42	micro RNA	maternal	promotes deadenylation of target mRNAs, degradation has not been shown	Wu et al., 2010
	miR-51-56	micro RNA	embryonic	promotes deadenylation of target mRNAs, degradation has not been shown	Wu et al., 2010
	miR-58/80-82	micro RNA	embryonic	promotes deadenylation of target mRNAs, degradation has not been shown	Wu et al., 2010
D. melanogaster	Smaug	RNA binding protein	maternal	binds to 3'UTR motif, recruits deadenylase complex	Semotok et al., 2005; Tadros et al., 2007
	Pumilio	RNA binding protein	embryonic	binds to target motif and destabilized mRNA	Thomsen et al., 2010
	miR-309	micro RNA	embryonic	miRNA mediated mRNA degradation	Bushati et al., 2008
	piRNAs	piwi-interacting RNAs	maternal	retrotransposon piRNA destabilizes maternal RNA (potentially in a complex with Smaug)	Rouget et al., 2010
M. musculus	ZFP36L2	zinc-finger, RNA binding protein	maternal	deadenylates maternal mRNAs	Ramos et al., 2004; Ramos, 2012
X. laevis	EDEN-BP	RNA binding protein	maternal*	triggers deadenylation by binding to 3'UTR cis element	Paillard et al., 1998
	miR-18	micro RNA	embryonic	maternal RNA degradation in somatic cells	Koebernick et al., 2010
	miR-427	micro RNA	embryonic	deadenylates and destabilzes maternal mRNAs	Lund et al., 2009
D. rerio	miR-430	micro RNA	embryonic	deadenylates and destabilzes maternal mRNAs	Giraldez et al., 2006
* in *X. laevis*, maternal mRNA degradation depends on embryonically transcribed factor					

binding to motifs in its target 3' UTR. Deadenylation occurs at developmental time points in which embryonic transcription is still absent (Paillard et al., 1998). In *X. laevis*, maternal mRNA degradation generally does not occur before the onset of embryonic transcription, indicating the involvement of an embryonically transcribed factor in the degradation process.
In *C. elegans*, the zinc-finger proteins MEX-5 and MEX-6 act together to degrade *nos-2* mRNA in somatic blastomeres (D'Agostino et al., 2006). Whether MEX-5/6 directly or indirectly lead to *nos-2* degradation remains, however, unclear.

First evidence for micro RNA (miRNA) mediated maternal mRNA degradation came from studies in the zebrafish *Danio rerio* (*D. rerio*). Microarray experiments showed that the miRNA *miR-430* directly regulates the degradation of hundreds of mRNAs (Giraldez et al., 2006). *miR-430* itself is among the earliest embryonically transcribed RNAs and its target mRNAs are enriched for maternally inherited RNAs which are rapidly degraded at the onset of embryonic transcription. Since this study, several maternal mRNA degrading miRNAs have been identified in different model organisms. For example, *X. laevis miR-427* deadenylates and destabilized maternal mRNAs in the early embryo and *miR-18* specifically degrades germline specific mRNAs which are mislocalized to somatic cells (Koebernick et al., 2010, Lund et al., 2009). In *C. elegans*, *miR-35-42*, *miR-51-56* and *miR-58/80-82* families are expressed in early embryos and have been shown to deadenylate target mRNAs (Wu et al., 2010). Whether this deadenylation leads to mRNA degradation *in vivo* remains, however, elusive. miRNA mediated maternal mRNA degradation has also been shown in *D. melanogaster*. *miR-309* is embryonically expressed and destabilizes several hundred mRNAs (Bushati et al., 2008). Interestingly, in the fly piRNAs (piwi-interacting RNAs) play a role in maternal mRNA degradation and might act together with the RNA binding protein Smaug to recruit the CCR4/POP2/NOT-deadenylation complex (Rouget et al., 2010).

Although factors and regulatory pathways of massive maternal mRNA degradation have been identified in most model systems, the precise function of mRNA degradation remains speculative. Hints are coming from work in *M. musculus* and *D. melanogaster* in which Gene Ontology (GO) term analysis indicated an enrichment of cell cycle related factors amongst the unstable maternal transcripts (Tadros et al., 2007, Hamatani et al., 2004). Indeed, if degradation of two fly Cdc25 homologues is impaired, embryos exhibit an abnormal additional mitotic cycle before cellularization (Edgar and Datar, 1996). Additionally, degradation of uniformly distributed maternal mRNA allows spatially and temporally restricted expression of their embryonic equivalents, as has been shown in *D. melanogaster* (De Renzis et al., 2007).

Recent studies further suggest that next to maternal mRNAs also maternal proteins are degraded. Main evidence comes from work in *C. elegans* in which the kinase MBK-2 was identified as a main regulator of maternal protein degradation. *mbk-2* mutant embryos fail to degrade meiosis and germ line specific proteins and do not initiate the first mitotic cell cycle (Pellettieri et al., 2003). In *X. laevis*, CPEB (cytoplasmic polyadenylation-element-binding protein) is degraded shortly before the first meiotic division and ectopic CPEB expression interferes with meiotic progression and mitosis (Mendez et al., 2002). In *C. elegans* and *D. melanogaster*, germ line specific proteins are actively degraded in the somatic blastomeres of the early embryo. This degradation most likely involves ubiquitin-dependent proteasomal degradation (reviewed in DeRenzo and Seydoux, 2004).

1.2.2. Embryonic gene activation

The second major process of MET leads to embryonic gene activation. The onset of EGA is a critical event in early embryogenesis and marks the first detectable gene expression within the embryo. It is believed that EGA occurs in successive, yet partially overlapping, waves (reviewed in Zurita et al., 2008, Tadros and Lipshitz, 2009). Initially, only a few genes are expressed but transcript abundance increases while embryonic development progresses

(Figure 3). In mouse, for example, first transcripts are detectable at the one cell stage embryo, mostly coming from the paternal pronucleus. In *C. elegans*, EGA is initiated at the four-cell stage but restricted to somatic cells, whereas in *D. melanogaster* and *X. laevis* embryonic transcription starts only after 8 and 6 cleavage cycles, respectively.

EGA onset is highly regulated by a variety of factors (Table 2). Among these factors are epigenetic modifiers. For example, BRG1 and SNF2H, ATPase subunits of the SWI-SNF complex and member of the ISWI family, respectively, accumulate at transcriptionally active sites of early mouse embryos. Together with TIF1α, they are responsible for accurate expression of a subset of early genes (Torres-Padilla and Zernicka-Goetz, 2006, Bultman et al., 2006). Also, frog DNA methyltransferase xDnmt1 is needed for accurate timing of EGA initiation, as depletion of xDnmt1 leads to precocious embryonic transcription. However, repression of EGA does not require the DNA methyltransferase domain (Stancheva and Meehan, 2000, Dunican et al., 2008).

Naturally, transcription factors regulate the onset of EGA. In *X. laevis* for example, the transcription factor VegT is necessary and sufficient for the expression of several early mRNAs (Skirkanich et al., 2011).
In *D. melanogaster*, Zelda has been identified as a key activator of very early embryonic transcription. Zelda binds to specific DNA motifs in the promoter region of its target genes which leads to active transcription of its targets (Liang et al., 2008). Recent chromatin IP data from different developmental time points suggest an even broader and more fundamental function of Zelda during all stages of EGA (Harrison et al., 2011). Interestingly, among the early Zelda target genes is miR-309 which is crucial for maternal mRNA degradation (discussed in chapter 1.2.1.). Therefore, depletion of Zelda not only leads to downregulated embryonically transcribed RNA but also to elevated levels of maternally inherited RNAs (Liang et al., 2008).

Table 2: Factors regulating EGA during MET

organism	factor name	factor type	activator/repressor of EGA	functions in…	known function	reference
C. elegans	PIE-1	zinc finger protein	repressor	embryonic germline precursor cells, starting at 4-cell stage (P2)	binds to P-TEFb subunit CycT1, thereby inhibiting CDK9 phosphorylation of PolII	Seydoux et al., 1996; Batchelder et al., 1999; Zhang et al., 2003
	OMA-1/2	zinc finger protein	repressor	1- and 2-cell embryo	binds TAF-4, keeps it in the cytoplasm	Guven-Ozkan et al., 2008
	TAF-4	TF	activator	somatic cells of 4-cell embryo	TFIID - part of the PolII pre-initiation complex	Guven-Ozkan et al., 2008
	GLD-1	RNA binding protein	repressor	central germ line	translational repressor, amongst its targets: maternal class I and II mRNA	Ciosk et al., 2006; Biedermann et al., 2009
	CYE-1	cyclin E	activator	*gld-1* dependent teratoma formation	necessary for mitotic entry	Biedermann et al., 2009
	FCP-1	CTD Ser5 dephosphatase	activator	mature oocyte and pre-EGA embryo	dephosphorylates PolII Ser5, thereby maintaining a pool of transcriptional competent PolII complexes	Walker et al., 2007
	UBA-1, UBC-2	ubiquitilation PW: E1, E2	activator	mature oocyte and pre-EGA embryo	unknown, seems to be necessary for maintaing a pool of transcriptional competent PolII complexes	Walker et al., 2007
D. melanogaster	Zelda	TF	activator	embryo, starting at cycle 8	direct binding to promoter elements of target genes	Liang et al., 2008; Harrison et al., 2011
	grainyhead	TF	repressor	embryo, potentially starting at 0 h of development	direct binding to promoter elements of target genes	Harrison et al., 2010
	STAT92E	TF	activator	embryo, MET-transition	direct binding to promoter elements of target genes	Tsurumi et al., 2011
	Smaug	RNA binding protein	activator	embryo, MET-transition	necessary for maternal RNA degradation during MET	Benoit et al., 2009
M. musculus	TIF1α	transcriptional regulator of nuclear receptors	activator/repressor	early zygote	recruited to specific sites, ensures localization and initiation of PolII and specific chromatin remodeling complexes	Torres-Padilla and Zernicka-Goetz, 2006
	SNF2H	ATPase subunite of ISWI complex	activator	early zygote	chromatin remodeling	Torres-Padilla and Zernicka-Goetz, 2006
	BRG1	ATPase subunite of SWI-SNF complex	activator	early zygote	chromatin remodeling	Torres-Padilla and Zernicka-Goetz, 2006; Bultman et al, 2006
	importin α7	facilitates nuclear translocation	activator	early zygote	unknown	Rother et al., 2011
	Mater	NALP protein family member	activator	early zygote	unknown	Tong et al., 2000
	Zar1	?	activator	probably pre-EGA zygote	unknown	Wu et al., 2003
	CycA2	cyclin	activator	early zygote	unknown	Hara et al., 2005
	NPM2	histone chaperone	activator	early zygote	unknown	Burns et al., 2003
X. laevis	VegT	transcription factor	activator	preMBT embryo	potentially: direct binding to promoter of target genes	Skirkanich et al., 2011
	β-catenin/ Xtcf3	Wnt signaling pathway	activator	preMBT embryo	potentially: direct binding to promoter of target genes	Yang et al., 2002
	xDnmt1	DNA methyl-transferase	repressor	preMBT embryo	unknown, NOT via it's DNA methylation catalytic domain	Stancheva and Meehan, 2000; Dunican et al., 2008

Of note, also Smaug as a key regulator of maternal mRNA degradation is essential for early EGA (Benoit et al., 2009). In addition to Zelda, the transcription factor grainyhead was identified to bind to similar DNA motifs. In contrast to Zelda, grainyhead acts as a repressor and is thought to compete with Zelda for DNA binding. This competitive interaction is proposed to work as a fine tuning of transcription (Harrison et al., 2010). The general transcription factor STAT92E has also been shown to activate, together with Zelda, a subset of Zelda target genes (Tsurumi et al., 2011).

In *C. elegans*, the transcription factor TAF-4 is essential for the expression of very early transcripts. As a transcription factor, TAF-4 functions in the nucleus but is kept in the cytoplasm by OMA-1/2 proteins. OMA-1/2 are degraded during the first two cell cycles leading to the release and nuclear translocation of TAF-4 at the four cell stage, the time when EGA starts in worms (Guven-Ozkan et al., 2008). In *C. elegans*, EGA is initiated only in the somatic blastomeres whereas the germ line precursor cell remains transcriptionally silent. This silent state is maintained by the zinc finger protein PIE-1 which binds to the CycT1 subunit of P-TEFb and thereby inhibits the phosphorylation and activation of Pol II (Seydoux et al., 1996, Zhang et al., 2003, Batchelder et al., 1999).

Cell cycle components are also implicated in regulating EGA onset. For example, CycA2 accumulates in mouse pronuclei before the onset of EGA. If nuclear accumulation is inhibited, cell cycle progression continues but EGA is strongly impaired (Hara et al., 2005). In *C. elegans*, depletion of the RNA binding protein GLD-1 leads to the formation of a germ line teratoma in which ectopic EGA occurs (Biedermann et al., 2009, Ciosk et al., 2006). In this background, cyclin E/CYE-1 is necessary for EGA initiation (Biedermann et al., 2009).

Additional activators of EGA include the Wnt signaling component β-catenin/Xtcf3 in *X. laevis*, importin α7, Mater, Zar1 and the histone chaperone NPM2 in *M. musculus*, as well as the Pol II dephosphatase FCP-1 and

components of the ubiquitilation pathway UBA-1 and UBC-2 in *C. elegans* (Yang et al., 2002, Rother et al., 2011, Tong et al., 2000, Wu et al., 2003, Burns et al., 2003, Walker et al., 2007).

In addition to transcriptional activators and repressors, the nucleo-cytoplasmic ratio affects the timing of EGA initiation. For example, polyspermic *X. laevis* embryos which have several paternal nuclei start EGA two cell cycles earlier than control embryos. The precocious onset of EGA is independent of cell cycle numbers and time after fertilization (Newport and Kirschner, 1982). It is therefore generally assumed that a maternally inherited EGA repressor is titrated away by the increasing number of nuclei compared to the relatively stable cytoplasmic volume.

Further, the “zygotic clock” model predicts that the time after fertilization or oocyte activation, independently of cell cycle events or the nucleo-cytoplasmic ratio, leads to early embryonic transcription. This model was initially suggested by studies in mouse embryos in which embryonic transcription starts at the same time after fertilization in embryos with blocked cytokinesis or blocked DNA replication, compared to control embryos (Bolton et al., 1984). Possible mechanistic explanations for the zygotic clock theory are coming from *C. elegans*. As described earlier, OMA-1/2 proteins sequester TAF-4 to the cytoplasm and only after their degradation TAF-4 is released and translocated into the nucleus where it initiates EGA. OMA-1/2 degradation is triggered by phosphorylation through MBK-2 (Guven-Ozkan et al., 2008). MBK-2 is maternally provided but activated only during oocyte maturation, shortly after which MBK-2 leads to global maternal protein degradation (Stitzel et al., 2006). Thus, OMA-1/2 degradation and subsequent TAF-4 dependent EGA are initiated during oocyte maturation and the entire process serves as a timer for EGA onset.

Taking together, maternal RNA and protein degradation and embryonic gene activation lead to the maternal-to-embryonic transition. Both processes are highly regulated and involve various maternally provided factors but also

embryonically transcribed genes. Both, maternal RNA degradation and EGA are intermingled, as shown for *D. melanogaster* factors Smaug and miR-309, and both processes in combination are necessary for successful completion of MET. Once MET took place, the embryo is in a position to control its own development independently of parentally provided factors.

1.3. Reproductive modes and embryogenesis in nematodes

The phylum of nematodes comprises several thousand species among which both, sexual reproduction and parthenogenesis are widespread. A very intensively studied nematode is *Caenorhabditis elegans* (*C. elegans*) which due to its size of about 1mm, its short reproductive cycle of about three to four days, its transparency and easiness to handle became a popular model system. *C. elegans* is a self-fertilizing hermaphrodite and thus reproduces sexually. Closely related species, however, have been described to reproduce parthenogenetically.

1.3.1. *C. elegans* reproduction

As a hermaphrodite, a single *C. elegans* worm produces both sperm and oocytes and thus is able to generate viable offspring without an interaction partner. Hence, *C. elegans* uses the long-term advantages of sexual reproduction but nonetheless has a high risk of suffering from inbreeding depression (see chapter 1.1.3.).

Hermaphrodite worms have two U-shaped gonad arms and produce sperm during the last of the four larval stages (L1 to L4). Sperm is stored in the spermatheca between the proximal gonad arms and the shared uterus of the worm. During adulthood, hermaphrodites produce oocytes in an assembly-line fashion until the end of their reproductive life. Oocytes are fertilized by the stored sperm and give rise to embryos which initially reside in the worm uterus and later are laid to the surrounding environment (Figure 4).

Next to hermaphrodites, males exist due to spontaneous chromosome missegregation - hermaphrodites inherit two sex chromosomes (XX) whereas males only have one X chromosome (X0). Male worms mate with hermaphrodites and, if both hermaphrodite and male sperm is present in the spermatheca, the male sperm is preferentially used for fertilization.

As a sexually reproducing species, both oocyte and sperm contribute to early embryogenesis.

1.3.1.1. *C. elegans* reproductive system

In *C. elegans*, a germ line precursor cell - the P blastomere - is already set aside during the very first embryonic cell division. Germ line specific proteins and mRNAs are specifically segregated into this one blastomere. The P blastomere gives rise to new germ lines and thus the “germ line information” is trans-generationally continuously maintained. The P1 blastomere divides and gives rise to a somatic blastomere and the P2 cell. This division pattern continues until the germ line founder cell P4 is formed. P4 divides once to give rise to the primordial germ cells Z2 and Z3. At the point of hatching, Z2 and Z3 are flanked by the two cells Z1 and Z4 which will form the somatic

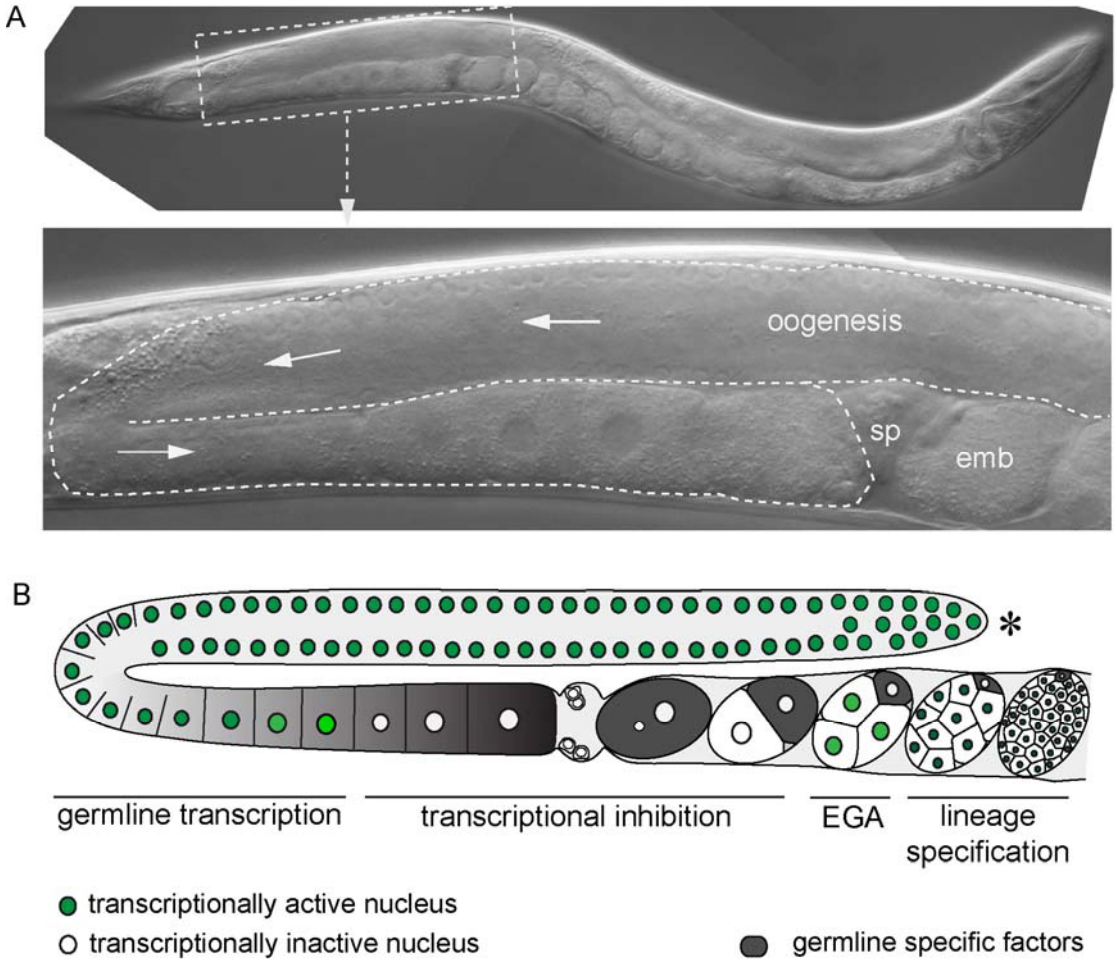


Figure 4: *C. elegans* reproductive tract
(A) Live image and (B) schematic representation of one of the two gonad arms of the adult *C. elegans* reproductive tract.
sp - spermatheca; emb - embryo

germ line. Beginning at the mid-L1 stage, the Z1 and Z4 cells divide and give rise to two distal tip cells (DTCs) and further somatic gonadal tissue. At the L3 larval stage, both gonad arms of a hermaphrodite rapidly extend and develop into the U-shape form. At the same time, germ cells proliferate and also meiosis is initiated. Gametogenesis now leads to the production of sperm during the L4 larval stage and oocytes during adulthood (reviewed in Hubbard and Greenstein, 2005).

The reproductive germ line contains proliferating stem cells at the distal part. Stem cell proliferation is induced by the DTC which sits on top of the germ line as part of the somatic gonad. The DTC secretes the Notch ligand LAG-2 that activates the Notch receptor GLP-1 which is expressed on the distal germ cells (Henderson et al., 1994, Crittenden et al., 1994). The activated Notch signaling pathway leads to mitotic proliferation. While the proliferating stem cells move away from the distal gonad, they are out of reach of LAG-2, initiate meiosis and differentiate into gametes. Stem cells are further maintained by the *C. elegans* FBF/Pumillio members FBF-1 and FBF-2 (Crittenden et al., 2002). FBF-1/2 are conserved RNA binding proteins which in the distal part of the gonad repress the cyclin E/Cdk2 inhibitor CKI-2 and therefore promote cell proliferation (Kalchhauser et al., 2011).

During the L4 larval stage, gametogenesis leads to the production of sperm. Primary spermatocytes initially reside in a syncytium but cellularize at the beginning of meiosis. During meiosis I, primary spermatocytes develop into haploid secondary spermatocytes which shortly after undergo meiosis II to form spermatids. Spermatids are transcriptionally silent but develop further into motile, fertilization competent spermatozoa. Unlike most vertebrate spermatozoa, *C. elegans* sperm does not posses a flagellum but forms a pseudopod with which it is able to crawl (reviewed in L'Hernault, 2006).

C. elegans sperm carries centrioles which it contributes to the embryo where it is necessary for mitotic spindle formation. Further, several sperm specific proteins have been identified. For example, the major sperm protein (MSP) is the highest abundant protein in sperm but actually consists of around 50

different proteins of a highly identical gene family (Ward et al., 1988, Klass and Hirsh, 1981). MSP is an important cytoskeletal protein and responsible for sperm motility (Italiano et al., 1996). Moreover, MSP has a crucial function as an extracellular signaling protein to induce oocyte maturation and ovulation (Miller et al., 2001). Most of the known sperm specific proteins are important during spermatogenesis to ensure correct sperm development and fertilization competence. For example, mutants lacking the FER-1 protein phenotypically develop a very short pseudopod and are unable to fertilize oocytes (Ward et al., 1981). Mutations in genes like *spe-9* lead to the development of mature sperm which, however, is unable to fertilize oocytes due to missing or impaired ligand-receptor bindings (Putiri et al., 2004). Over 40 genes are known to affect spermatogenesis and most of these are sperm specifically expressed. The majority of sperm defective mutants still express MSP so that oocyte maturation and ovulation is induced. As no fertilization takes place in these mutants, ovulated but unfertilized oocytes are produced which initially reside in the worm uterus (McCarter et al., 1999).

So far, only sperm defective *spe-11* mutants have been identified to be able to fertilize oocytes. SPE-11 protein is associated with sperm chromatin during late stages of spermatogenesis (Chu et al., 2006). However, it has no known function during spermatogenesis, yet it is necessary for accurate eggshell formation in the developing embryo (Johnston et al., 2010). Therefore, embryos derived from *spe-11* mutants lack a functional eggshell, undergo impaired meiosis II and progress through mitosis without cytokinesis (McNally and McNally, 2005). However, failure of meiotic chromosome segregation and cytokinesis defects, including polar body extrusion, might be directly linked to a non functional eggshell (reviewed in Johnston and Dennis, 2011). *spe-11* mutant embryos can be rescued by providing functional SPE-11 protein through oocytes (Browning and Strome, 1996). Further, studies of mutants in which sperm loses its DNA during late spermatogenesis revealed that the sperm pronucleus is not necessary for early embryonic development (Sadler and Shakes, 2000). Both findings indicate that SPE-11, despite being

localized to sperm chromatin, is unlikely to have a second function in embryogenesis through sperm DNA modifications.

C. elegans sperm also carries mRNAs which are inherited to the embryo although precise numbers and gene products are not known. One study, however, showed that the transcription factor ELT-1 regulates gene expression during spermatogenesis. By *in situ* hybridization against the *elt-1* mRNA, it could be shown that *elt-1* is enriched in mature sperm and thus most likely is inherited to the embryos (del Castillo-Olivares et al., 2009). Whether *elt-1* inheritance is a byproduct of its expression during spermatogenesis or whether it has a function in early embryogenesis remains elusive.

After the L4-to-adult molt, hermaphrodites switch from spermatogenesis to oogenesis, a process which is highly regulated. Terminal regulators of the sperm/oocyte switch seem to be FOG-1 and FOG-3 which promote sperm differentiation and therefore have to be repressed to allow oogenesis (Barton and Kimble, 1990, Ellis and Kimble, 1995). FOG-1 and FOG-3 expression are directly regulated by FBF/Pumillio and TRA-1 (Thompson et al., 2005, Chen and Ellis, 2000). TRA-1 seems to be repressed by the *fem* genes FEM-1, FEM-2 and FEM-3 which in turn are regulated by the oogenesis promoting protein TRA-2 (Hodgkin, 1986). Taken together, FEM-1/2/3 and FOG-1/3 expression promotes spermatogenesis whereas TRA-1/2 and FBF/Pumillio promote oogenesis. These factors make up the core regulators of the sperm/oocyte switch but additional genes are known to influence sex determination at various levels (reviewed in Ellis and Schedl, 2007).

During adulthood, *C. elegans* hermaphrodites produce oocytes. Germ cells enter meiosis and reside in a syncytium. While they move from the more distal part of the gonad to the proximal part, individual nuclei progress through meiotic prophase I. Around the bend region of the gonad arm, nuclei begin to cellularize and form oocytes. Oocytes grow and accumulate yolk lipoproteins through endocytosis (Hall et al., 1999). During most of their development, germ cells are transcriptionally active and produce mRNAs and proteins for

their own need and for storage in the oocyte. In the proximal gonad arm, transcription ceases which coincides with oocyte maturation, but does not depend on it (Walker et al., 2007).

As in other species, mature *C. elegans* oocytes are loaded with proteins and mRNAs which are necessary to drive early embryonic development (see chapter 1.1.2.1. and 1.2. for specific examples).

In the maternal *C. elegans* germ line, gene expression is regulated primarily post-transcriptionally by binding of RNA binding proteins to 3' UTRs (Merritt et al., 2008). Several transcriptional repressors are known of which GLD-1 plays a fundamental role during oogenesis with respect to early embryogenesis. GLD-1 is a member of the signal transduction and activation of RNA (STAR) family of RNA binding proteins and is expressed in the central germ line. It is involved in sex determination, the mitosis-to-meiosis switch and maintenance of germ cell identity (Ciosk et al., 2006, Francis et al., 1995, Biedermann et al., 2009). GLD-1 binds to a degenerated RNA motif in the 5' and 3' UTRs of a large number of germ line expressed mRNAs (Wright et al., 2010, Jungkamp et al., 2011). Target genes of GLD-1 include various maternally provided mRNAs which are necessary for several stages of embryonic development (Wright et al., 2010, Scheckel et al., 2012). For example, GLD-1 inhibits the translation of *pal-1* mRNA which is a transcription factor essential for muscle development in the embryo. As GLD-1 is only present in the central gonad, a second RNA binding protein, MEX-3, binds and represses *pal-1* mRNA in the proximal germ line (Mootz et al., 2004). Interestingly, *mex-3* mRNA itself is a target of GLD-1 which demonstrates that translational repression is hierarchical and correct expression of RNA binding proteins determines spatial repression of at least a subset of target mRNAs.

If GLD-1 is depleted, germ cells enter meiosis but are not able to progress through meiotic prophase I. Instead, they re-enter the mitotic cell cycle, proliferate, express early embryonic genes and differentiate into somatic cells to give rise to a germ line teratoma (Biedermann et al., 2009, Ciosk et al.,

2006). Teratoma formation enhances if in addition to GLD-1 also MEX-3 is depleted (Ciosk et al., 2006). Re-entry into mitosis is necessary for expression of early embryonic genes and general teratoma formation, and in turn depends on cyclin E/CYE-1 expression. In wild type animals, *cye-1* mRNA is repressed by GLD-1 and thus GLD-1 inhibits mitotic re-entry and allows progression through meiosis and oocyte formation (Biedermann et al., 2009).

1.3.1.2. *C. elegans* maternal-to-embryonic transition

The most proximal oocyte arrests in diakinesis of meiotic prophase I but matures upon sensing the sperm derived MSP protein (Miller et al., 2001, McCarter et al., 1999). During maturation, nuclear envelop breakdown is initiated and the oocyte progresses to meiotic metaphase I. In parallel, gonadal sheath contractions intensify and the oocyte is squeezed into the adjacent spermatheca (McCarter et al., 1999). Shortly after ovulation into the spermatheca, a single calcium wave occurs which spreads through the entire oocyte. The starting point most likely coincides with the sperm entry point, although a direct fusion between sperm and oocyte could not be observed due to technical limitations (Samuel et al., 2001). In the absence of fertilization-competent sperm, maternal nuclei terminate meiosis at anaphase I, whereas fertilized oocytes will progress through meiosis I and II (McNally and McNally, 2005).

During oocyte maturation, cortical rearrangement leads to the formation of a basal lamina, and shortly after fertilization eggshell formation begins. Initially, the vitelline layer is formed as a modified basal lamina which acts as a polyspermy barrier. Underneath the vitelline layer, the chitin layer is synthesized and protects the developing embryo from mechanical and chemical stress. The final lipid-rich layer forms underneath the chitin layer and creates the correct osmotic pressure to form the extra embryonic matrix between cell membranes and eggshell and thus ensures embryonic development (reviewed in Johnston and Dennis, 2011). Chitin synthesis initiates at the point of sperm entry and requires SPE-11 for spreading around the oocyte (Johnston et al., 2010). Embryos from which the vitelline and chitin

layers have been removed are viable and will hatch whereas destruction of the lipid-rich layer leads to embryonic arrest (Schierenberg and Junkersdorf, 1992). Although both the vitelline and chitin layers are needed to establish the functional lipid-rich layer, these findings demonstrate that only the latter is essential for embryonic development.

Fertilization and the sperm derived centrosome further initiates the anterior-posterior (A-P) axis in early embryos (Cowan and Hyman, 2004). Actomyosin cortex contractions lead to general cortical ruffling. During this ruffling process, centrosomes associated with the paternal pronuclei localize to the posterior whereas the maternal pronucleus localizes to the anterior part of the embryo. Cortical ruffling is also essential to asymmetrically distribute PAR proteins which define the A-P axis in early embryos (Munro et al., 2004, Kemphues et al., 1988, Morton et al., 2002). PAR-3 and PAR-6 become restricted to the anterior cortex, PAR-1 and PAR-2 are localized to the posterior cortex and PAR-4 and PAR-5 are uniformly distributed (reviewed in Gonczy and Rose, 2005). Establishment of the A-P axis is crucial for the segregation of cell fate determinants towards either the anterior or posterior part of the one-cell embryo. For example, germ line specific P granules as well as PIE-1 move towards the posterior end and are, in addition, degraded locally at the anterior end (Strome and Wood, 1983, Mello et al., 1996).
After A-P polarity is established, pronuclear fusion takes place and asymmetric mitotic cell division forms two daughter cells which are unequal in size and cellular components. The anterior cell undergoes a symmetric division perpendicular to the A-P axis, whereas the posterior P1 cell continues to divide asymmetrically (reviewed in Gonczy and Rose, 2005).

During these early cleavages, maternally inherited mRNAs exhibit two distinct localization patterns. Maternal class I mRNAs like *tbb-2* and *dpy-30* are uniformly distributed whereas maternal class II mRNAs like *cey-2* and *pos-1* become restricted to the posterior P-lineage (Seydoux and Fire, 1994). Approximately 30 % of maternally inherited mRNAs are degraded in the early *C. elegans* embryo. Many of these degraded mRNAs remain at low levels

which might reflect the presence of maternal class II transcripts in germ line precursor cells (Baugh et al., 2003). After EGA onset, genes belonging to the maternal class I, are additionally newly synthesized in embryonic cells.

At the four-cell stage, EGA starts and several early embryonic transcripts, including *vet-1*, *vet-4*, *vet-6* and *pes-10,* are detected (Seydoux et al., 1996). The onset of EGA in *C. elegans* is regulated by OMA-1/2 proteins which keep the essential TFIID complex member TAF-4 in the cytoplasm but are themselves degraded upon MBK-2 phosphorylation (see chapter 1.2.2. for details) (Guven-Ozkan et al., 2008). Next to TAF-4, the TFIID components TAF-10 and TAF-11 are important for the activation of a subset of embryonically transcribed genes (Walker et al., 2001). Embryonic transcript levels increase after EGA onset and reach a plateau stage at the beginning of gastrulation, indicating the completion of MET (Baugh et al., 2003). In *C. elegans*, gastrulation begins at the 26-cell stage but embryos depleted for the Pol II subunit AMA-1 arrest at around the 100-cell stage which leads to the assumption that the MET is completed later (Powell-Coffman et al., 1996). However, AMA-1 depleted embryos are gastrulation defective and proliferation might simply continue until maternally provided factors are exhausted.

Notably, EGA only occurs in somatic precursor cells due to PIE-1 mediated transcriptional repression in germ line blastomeres (see chapter 1.2.2.). Initially, A-P axis formation and subsequent asymmetric segregation and local degradation of germ line specific factors define the somatic versus germline fate of early blastomeres. Later during *C. elegans* development, the germ line-soma distinction is maintained through a poorly understood mechanism which involves several chromatin remodeling complexes. In all eukaryotic cells, the DNA is wrapped around protein octameres called histones. The histones H2A, H2B, H3 and H4 make up the core histones and are present twice in each octamere. Histone tails are subject to reversible posttranslational modifications that alter their interactions with other proteins and thereby influence the expression status of nearby genes. For example, tri-methylation

of the lysine residue 27 on histone 3 (H3K27m3) is associated with gene silencing whereas methylation of histone 3 lysine residue 36 (H3K36m) is associated with active gene expression. In *C. elegans*, germ line development depends on several *mes* genes which were initially identified as maternal effect sterile genes because progeny of homozygous *mes* mutants have strong defects in germ line development and are sterile (Capowski et al., 1991). The *mes* genes have been identified as the *C. elegans* H3K36 methyltransferase MES-4 and the *C. elegans* polycomb repressive complex 2 (PRC2) which consists of MES-2, MES-3 and MES-6 and is responsible for repressive H2K27m3 marks (Holdeman et al., 1998, Bender et al., 2006). Both MES-4 and PRC2 are essential for X-chromosome silencing in the adult germ line but have additional roles during embryogenesis to promote trans-generational epigenetic memory of germ line expressed genes and differentiation, respectively (Garvin et al., 1998, Bender et al., 2006, Yuzyuk et al., 2009, Rechtsteiner et al., 2010, Furuhashi et al., 2010). In addition, a specialized *C. elegans* nucleosome remodelling and histone deacetylase (NuRD) complex has been shown to repress germ line specific genes in somatic tissues (Unhavaithaya et al., 2002). In *C. elegans*, several NuRD complexes have been described, among them the specialized MEP-1 containing (MEC) complex which consists of MEP-1, the histone deacetylase HDA-1 and the Mi-2 orthologue LET-418 (Passannante et al., 2010). If components of the MEC complex are depleted from worms, germ line specific genes are expressed in somatic tissues and worms do not develop past the L1 stage (Passannante et al., 2010, Unhavaithaya et al., 2002). As the MEC components interact with the transcriptional repressor and germ line determinant PIE-1, but MEC mutant phenotypes are rescued by mutating *mes* genes, both chromatin remodelers are believed to be directly involved in maintaining the germline-soma distinction (Unhavaithaya et al., 2002). A detailed mechanism of how the complexes interact is not known, but it is proposed that in germ line precursor cells PIE-1 inhibits the MEC complex and active *mes* genes promote a germline pattern of gene expression whereas in somatic cells the active MEC complex inhibits *mes* genes which leads to a somatic pattern of gene expression (reviewed in Strome, 2005).

Shortly after onset of EGA at the four cell stage, differentiation and lineage specification begins in the embryo. In *C. elegans*, lineage specification primarily depends on spatially and timely expression of lineage driving transcription factors. The earliest lineage to arise is the intestine. At the four-cell stage, the ventral EMS cell divides and gives rise to the gut founder blastomere - the E cell. Gut fate is initiated by the maternally provided transcription factor SKN-1 which transiently activates the GATA-type transcription factors END-1 and END-3 (Zhu et al., 1998, Zhu et al., 1997). END-1/3 activate additional GATA-type transcription factors, among them the essential *elt-2* gene (Fukushige et al., 1998). ELT-2 expression is first detected in the 2E cells (after the first division of the E cell) and is maintained through auto-activation in every cell of the developing and adult gut (Fukushige et al., 1999). As an essential transcription factor, ELT-2 expression leads to the activation of numerous target genes which function during gut differentiation and regulate gut functions (McGhee et al., 2009). Ectopic expression of ELT-2 in non-gut blastomeres of the early embryo reveals its high potential to induce intestinal differentiation. All cells develop into gut cells and eventually express terminal gut differentiation factors like IFB-2 (Fukushige et al., 1998). The latter experiment of inducing a cell fate switch in embryonic blastomeres generally demonstrates the high embryonic plasticity. Overexpression of a variety of other transcription factors including muscle, hypodermal and epithelial induction, confirmed this high cellular plasticity of early embryonic cells (reviewed in Zuryn et al., 2011). Ectopic reprogramming succeeds only during a narrow time window of early embryonic development in which blastomeres have not yet fully adopted their wild type cell fate (Fukushige and Krause, 2005, Gilleard and McGhee, 2001). Therefore, induced cell fate reprogramming can be used to identify the developmental stage of embryonic cells.

1.3.2. Parthenogenesis in nematodes

Several nematode species have been described to reproduce parthenogenetically. For example the parasitic nematode *Strongyloides ratti* exhibits a generational switch of sexual reproduction and parthenogenesis (Viney, 1999). Of the parthenogenetically reproducing free soil living nematode species, *Acrobeloides nanus* (*A. nanus*), *Diploscapter coronatus* (*D. coronatus*) and *Plectus* sp. have been studied in more detail.

As in *C. elegans*, elevated levels of active MAP kinase are detectable in most proximal oocytes of *A. nanus* and *D. coronatus* (Heger et al., 2010). In *C. elegans*, MAP kinase activation occurs as part of the oocyte maturation process in response to sperm MSP signaling (Miller et al., 2001). Studies in parthenogenetically reproducing nematodes revealed that MSP is not expressed despite the presence of functional *msp* genes (Heger et al., 2010). Thus, oocyte maturation is initiated by different, yet unknown mechanisms.

D. coronatus and *A. nanus* restore diploidy levels by an abnormal meiosis. In the case of *A. nanus*, terminal fusion of the second polar body and the oocyte nucleus has been identified. *Plectus* sp. on the other hand, undergoes normal meiosis, but nuclear growth before the first mitotic cell division suggests gamete duplication to take place (see chapter 1.1.2.2.) (Lahl et al., 2006).

In *C. elegans*, A-P axis formation depends on the sperm entry point with the posterior pointing towards the vulva. *A. nanus* embryos also show a preferred A-P axis orientation but with the posterior part pointing towards the proximal gonad arm. Embryos from *D. coronatus* exhibit a random A-P axis formation. Polarity in *Plectus* sp. could not be analyzed as embryos do not develop *in uteru* (Lahl et al., 2006). Therefore, in parthenogenetically reproducing nematodes, A-P axis formation does not necessarily depend on external cues like sperm entry in *C. elegans*.

Further differences involve the supply of maternal factors to the early embryo. If embryonic transcription is blocked, *C. elegans* embryos divide until around

the 100 cell stage whereas *A. nanus* embryos arrest at the five-cell stage (Powell-Coffman et al., 1996, Laugsch and Schierenberg, 2004). These findings show an absolute requirement for embryonic transcripts in *A. nanus* early embryogenesis and indirectly suggest differences in the amount of maternally inherited factors between both species. Direct comparisons between *C. elegans* and any parthenogenetically reproducing nematode species with regard to the oocytic transcriptomes and proteomes have not been performed. Further, mechanisms leading to mitotic spindle formation despite the lack of sperm contributed centrioles are unknown.

All three parthenogenetically reproducing worm species lay their embryos as a soft-shelled 1-cell stage embryo into the surrounding environment. The embryos are severely squeezed while passing through the vulva and only afterwards develop a functional eggshell (Lahl et al., 2006). It remains, however, speculative whether the squeezing leads to oocyte activation as reported in flies or whether the environmental change itself is needed for e.g. releasing the oocyte from a maternal repressor of oocyte activation.

1.4. Scope of the thesis

At the start of my thesis, it was known that true parthenogenesis does not occur in *C. elegans*. However, as in other species, *C. elegans* oocytes were known to possess a large amount of factors essential for early embryogenesis. Unfertilized oocytes have been described to partially continue meiosis and to endoreplicate. These findings led to the assumption that unfertilized oocytes are more similar to embryos than to germ cells, but no detailed analysis on embryonic-like development in unfertilized oocytes has ever been performed. In addition, it became evident that genes which are only expressed in early embryos are also found in the developing teratoma of an all maternal background. This suggests that teratoma formation might be caused by premature embryonic-like development but further in depth analyses were also missing.

My thesis aimed to analyze the embryonic-like development in unfertilized oocytes and the teratoma. What are the parallels and differences in the "development" of these mutants? Why does embryonic-like development occur in the teratoma but apparently not in unfertilized oocytes? Are there unknown restrictions to parthenogenesis?

It turned out that teratoma formation shows all characteristics of early embryogenesis, including embryonic gene activation and differentiation, whereas unfertilized oocytes exhibit early features of embryogenesis but fail to differentiate. The reasons for the inability to differentiate were further investigated and I will present our current understanding of how fertilization licenses the competence of lineage specific transcription.

2. Experimental procedures

Nematode culture

Standard procedures were used for maintaining strains. Animals were grown at 20 °C unless stated otherwise. Strains carrying temperature sensitive alleles were maintained at permissive temperatures (15 ° C for *emb-27*, 20 °C for *fem-1*) but shifted to restrictive temperatures (25 °C) for experiments. Synchronous populations were obtained by collecting embryos from bleached adults and synchronizing larvae by starvation before feeding. RNAi experiments were performed by feeding animals with bacteria expressing RNAi clones from the Ahringer (*ama-1*, *mes-3, mes-6, mei-1*) and Open Biosystem (*mep-1, egg-3, fem-1, gna-2, sqv-4*) libraries. The *zen-4* RNAi clone was created by Rafal Ciosk and contains the nucleotides 1426 to 2148 of the zen-4 isoform A coding sequence, integrated into the empty pMD3 backbone.

Strain list

Table 3: List of *C. elegans* strains used for analyses

strain #	genotype	generated by / courtesy of
342	wild type (N2)	
668	[*vet-4 promoter::NLS-GFP::vet-4 3' UTR*]	microparticle bombardment
463	*mes-4 (bn85)*/DnTIG.	Susan Strome
705	*mes-4 (bn85)*/DnTIG; [*vet-4 promoter::NLS-GFP::vet-4 3' UTR*].	crossing #463 and #668
433	*mes-2(bn11) unc-4(e120)*/mnC1 *dpy-10(e128) unc-52(e444)*II.	CGC #SS186
669	*mes-2(bn11) unc-4(e120)*/mnC1 *dpy-10(e128) unc-52(e444)* (II); [*vet-4 promoter::NLS-GFP::vet-4 3'UTR*].	crossing #433 and #668
738	*let-418(ar114)/unc-46(e177) dpy-11(e224)* V.	CGC #GS402
867	*let-418(ar114)/unc-46(e177) dpy-11(e224)* V; [*vet-4 promoter::NLS-GFP::vet-4 3' UTR*].	crossing #867 and #668
121	*fem-1 (hc17); gld-1 (q485)*/ hT2[qIs48]	
356	*gld-1 (q485), mex-3 (or20)* / hT2[qIs48]	
552	*mex-3(or20) gld-1(q485)*/hT2[qIs48]; [*vet-4 promoter::NLS-GFP::vet-4 3' UTR*].	crossing #356 and #668
789	[*hsp16-25::elt-2::unc-54*]	James McGhee (strain JM57)

899	*gld-1 (q485), mex-3 (or20)* / hT2[qIs48]; [*hsp16-25::elt-2::unc-54*]	crossing #356 and #789
105	*fem-1(hc17)* IV.	CGC #BA17
1111	*fem-1(hc17)* IV; [*vet-4 promoter::NLS-GFP::vet-4 3' UTR*].	crossing #105 and #668
98	*pes-10::GFP* axIs36[pJH1.16,*dpy-20*]	Geraldine Seydoux
313	*fem-1(hc17)* IV; *pes-10::GFP* X	crossing #105 and #98
1018	kcIs21 [*ifb-2::cfp*]	Rudolf Leube (strain BJ52)
1043	[*hsp16-25::elt-2::unc-54*]; kcIs21 [*ifb-2::cfp*]	crossing #789 and #1018
1027	*spe-11(ok2143)* I/hT2[qIs48].	CGC #VC1741
1045	*spe-11(ok2143)* I/hT2[qIs48]; [*hsp16-25::elt-2::unc-54*].	crossing #1027 and #789
790	*unc-119(ed3)* III; teIs1[pRL475(*P(oma-1)oma-1::gfp*; + pDPMM016*(unc-119+)*].	CGC #TX189
1044	*emb-27(g48)* II.	CGC #GG84
1100	otIS305[*hsp::che-1*]; otIs264[*ceh-36::TagRFP*]; ntIs1[*gcy::gfp*]	Oliver Hobert

Generation of *vet-4* reporter strain (#668)

The plasmid for the *vet-4* reporter construct includes a 3 kb *vet-4* promoter fragment with an additional NLS fused to GFP open reading frame and the *vet-4* 3' UTR (500 bp downstream of the endogenous *vet-4* stop codon). The *vet-4* reporter plasmid was constructed using the MultiSite Gateway Three Fragment Vector Construction Kit (Invitrogen), primers are listed in table 4. The *vet-4* reporter plasmid was transformed into *unc-119(ed3)* animals by microparticle bombardment as previously reported (Wright et al., 2011, Praitis et al., 2001).

Table 4: Primers used for cloning the *vet-4* reporter strain (5' to 3'):

vet4-p1.3	GGGGACAACTTTGTATAGAAAAGTTGATCTGTTTACCTGACAATATTTGGAC
vet4-p2	GGGGACTGCTTTTTTGTACAAACTTGTAACCTTTCTCTTCTTCTTCGCCATTATCTGAAATTAGAGTAATTAGGGTTTTGAAAATGG
vet4-p3	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCAGTAAAGGAGAAGAACTTTTCAC

vet4-p4	GGGGACCACTTTGTACAAGAAAGCTGGGTTTATTTGTATAGT TCATCCATGCCATG
vet4-p5	GGGGACAGCTTTCTTGTACAAAGTGGTTTATAATTGTTTGTT TTTCTACTTCC
vet4-p6	GGGGACAACTTTGTATAATAAAGTTGAATGAACATTGGCCAT TTCTGCGAAAAAAAAC

Immunofluorescence

Experiments were essentially performed as previously described (Lin et al., 1998) for antibodies against PIE-1 (dilution 1:10, courtesy of J. R. Priess) and IFB-2 (“MH33”, dilution 1:200, courtesy of J. McGhee). Staining against ELT-2 (dilution 1:1000, courtesy of J. McGhee) was performed as above but incubated 6 min in -20 °C methanol instead of dimethyl formamide. Stainings against P-Ser5 (“H14”, dilution 1:20, abcam) and P-Ser2 (“H5”, dilution 1:20, abcam) were performed as previously described (Seydoux and Dunn, 1997). Secondary antibodies used in this study were goat anti-mouse IgG alexa-568, goat anti-mouse IgG alexa-488, and goat anti-mouse IgM alexa-488 (Molecular Probes).

RNA in situ hybridization

RNA hybridization was performed as described by Broitman-Maduro and Maduro (http://www.faculty.ucr.edu/~mmaduro/resources.htm). Probes used in this study are against *vet-4* (Biedermann et al., 2009), *gfp* (nucleotides 1 - 714), *pos-1* (nucleotides 85 - 611), *cey-2* (nucleotides 210 - 683), *tbb-2* (nucleotides 723 - 1220) and *dpy-30* (nucleotides 4 - 360).

Image acquisition

Images were captured on a Zeiss AxioImager Z1 microscope equipped with AsioncamMRm REV CCD camera. All images were processed with Adobe Photoshop CS3 in an identical manner.

Heat shock experiments

Ectopic expression of heat shock driven transcription factors in embryos and ovulated oocytes was performed as described previously (Fukushige et al.,

1998). For gonadal expression, heat shock was performed for 60 min at 33°C on 1 day old animals (counting from L4-to-adult molt).

Embryos, unfertilized oocytes, and whole worms were allowed to recover from heat shock for 1 h to check for ELT-2 expression and for 6 h or 16 h (as indicated) to check for IFB-2 and neuronal marker expression.

emb-27 embryos for heat shock were obtained by crossing feminized (*fem-1* RNAi) *hsp16-25::elt-2::unc-54* carrying hermaphrodites with *emb-27* males which were treated as previously described (Sadler and Shakes, 2000).

RT-qPCR analysis

Quantification of mRNAs from dissected gonads of 1 day old worms was performed as previously described (Biedermann et al., 2009) but with adding 2 µg human carrier RNA to each sample before RNA extraction. Equal amounts of RNA were used in RT reactions and values were normalized to human RNA (hGAPDH).

For embryos and unfertilized oocytes, RNA was extracted as above with sample sizes of 10 embryos or unfertilized oocytes. Embryos were transferred into the extraction buffer and their eggshell damaged manually with a needle.

Table 5: Primers used for qPCR analysis (5' to 3'):

gene	primer	
act-1	forward	CTATGTTCCAGCCATCCTTCTTGG
	reverse	TGATCTTGATCTTCATGGTTGATGG
tbb-2	forward	GCTCATTCTCGGTTGTACCA
	reverse	TGGTGAGGGATACAAGATGG
vet-1	forward	AAAGAACTGAAACTATGTTTGCTG
	reverse	CTCTCGTCGTGTTTTCTGATG
vet-4	forward	AAGGATTTCACTGCTTGCTC
	reverse	CGTCGTTTTCGATTTCTCCG
vet-6	forward	GTGCGAGACAAGAATGTAATCC
	reverse	TTCTTGAACTCTTGGAACACAG
pes-10	forward	GCGATGATTTCATGATTTCCTG
	reverse	AATTTCGTAGTCAATCTGCTCC
hlh-1	forward	ACGATTATGTGACTTCCTCTC
	reverse	GATGATCTCTATCGTCGTCC

unc-120	forward	GGGTATTATGAAGAAGGCATTCG
	reverse	TGCATATGTGTAGACATGACCA
end-1	forward	GGGCAATACTTTGTTCAATCG
	reverse	GGATACTGTTGTGAGTAGCA
end-3	forward	GCCTATTAATGACCTCCAGC
	reverse	CCCGTCAATTGGTATCTCTG
elt-2	forward	AGTAAACGGAGGAATGATGTG
	reverse	CTGCTCTGAAGGTATTTCCA
pha-4	forward	CCAGAATTTCCTGAACAACAC
	reverse	GTTGGTGGAGCTGTAAAGAG
tbx-2	forward	AAGTGGAAGACGGATATTCC
	reverse	TTGTAACGGTGTTCATCAGC
elt-1	forward	AACTTCATAAGGTGGAACGT
	reverse	CTCTTTCTTCATTCTTCGCG
ifb-2	forward	GATTGCTGAACTTCAAGCTC
	reverse	ACATGAGTGAATCTTCCTTCC
asp-1	forward	AAGTTCTCGATCCAATACGG
	reverse	AAACTCCGAACTCTTGAGAC
haf-9	forward	AAGTAGTAACAGCAGTATTCGG
	reverse	ACCAAATACAGTTGAAGCCA
clec-85	forward	ACCTGTGCTACTCAATTTCC
	reverse	GAGGGAGGTATGAATCACTG
sbp-1	forward	TGGAAGATGAGCAATTATCCC
	reverse	TGTAAAGATCTCGTCACCAC
cpr-1	forward	CAATCTGGATATTCAACTGCC
	reverse	GTAGATTTCAGCTTGGATGGA
hGAPDH	forward	GGAGTCAACGGATTTGGTC
	reverse	AAACCATGTAGTTGAGGTC

Statistical analysis

Statistical significance was detected by analyzing differences of means using T-tests of the SigmaPlot 11.0 software. Each T-test preceded a Shapiro-Wilk test to check for normal distribution of the data points. Single asterisks indicate $p<0.05$, double asterisks indicate $p<0.01$ and triple asterisks indicate $p<0.001$

3. Results

3.1. Chromatin modifiers maintaining the germ line - soma distinction have no additional role in EGA onset

Epigenetic modifications have well characterized regulatory functions during embryogenesis. Mouse and frog studies demonstrated the importance of chromatin modifiers and DNA methyltransferases on the onset of EGA (Torres-Padilla and Zernicka-Goetz, 2006, Bultman et al., 2006, Stancheva and Meehan, 2000). In contrast to vertebrates, however, *C. elegans* lacks methylated CpGs and conventional DNA methyltransferases, so that DNA methylation can be ruled out as an EGA regulator (reviewed in Bird, 2002). Chromatin modifiers, however, are well conserved and play fundamental roles during *C. elegans* development (reviewed in Cui and Han, 2007). The *C. elegans mes* genes, comprised of the *C. elegans* PRC2 complex members MES-2, MES-3 and MES-6 and the H3K36 methyltransferase MES-4, together with a specialized NuRD complex, the MEC complex, comprised of MEP-1, HDA-1 and LET-418, maintain the germ line - soma distinction during embryonic and larval development (see chapter 1.3.1.2.). All modifiers are expressed in the adult germ line and thus likely to be maternally inherited to the embryo. EGA in *C. elegans* is initiated at the four cell stage but only in somatic blastomeres which suggest a potential role of germline versus somatic determinants in the regulation of EGA onset. We therefore asked whether the *mes* genes and the MEC complex serve as EGA regulators.

The *vet-4* reporter strain mimics endogenous *vet-4* expression

In order to visualize EGA, we created a reporter strain for the early embryonic gene *vet-4*. *vet-4* mRNA is expressed in the three somatic blastomeres of a four cell stage embryo (Seydoux et al., 1996). *vet-4* transcript abundance mildly increases during the next 40 minutes of embryonic development after which ists expression is strongly enhanced. Following another 40 minutes of embryonic development, *vet-4* transcript abundance reaches a plateau until it strongly decreases at around 140 minutes after the four cell stage (Baugh et al., 2003). The function of the VET-4 protein is unknown, but domain structure

analysis suggests a function in ubiquitin-proteasome mediated protein degradation.

With the help of Mathias Senften, we created a *vet-4* reporter strain which contains a 3 kb promoter fragment (with two A-to-G point mutations at position 667 and 805) fused to a nuclear localization sequence (NLS), a single *gfp* open reading frame (ORF) and 500 bp downstream the *vet-4* ORF as a 3' UTR (Figure 5A). For *vet-4*, no endogenous 3' UTR is annotated and a 3' race experiment on the endogenous *vet-4* mRNA could not identify the presence of a functional 3' UTR (data not shown).

Worms carrying the *vet-4prom::gfp* reporter plasmid did not express any GFP in the germ line. GFP expression was limited to embryos and detectable in a few cells in L1 larvae. In embryos, GFP expression was weakly detectable at around the 20-cell stage after which it strongly increased until around the 100 cell stage (Figure 5B). GFP intensities varied between embryos of the same age. Despite a functional NLS signal, GFP was uniformly distributed in embryonic cells, which might be attributed to passive diffusion of the small GFP protein through nuclear pores. The onset of GFP expression did not correlate with the expected four to eight cell stage. *In situ* hybridization against the *gfp* mRNA, however, verified its expression at the four cell stage (Figure 5C). Further, like the endogenous *vet-4* mRNA, *gfp* transcripts were only expressed in somatic blastomeres and absent from the germ line precursor cells (Figure 5C and D). Thus, given that cells of the early embryo divide every 20 minutes, the delay in excitable GFP protein detection might be due to the time which the GFP protein needs to mature.

GFP expression exclusively depended on embryonic transcription as no GFP was detectable in embryos depleted for the Pol II subunit AMA-1 (Figure 5E).

In summary, the *vet-4* reporter strain mimicked embryonic *vet-4* expression.

mes and MEC complex genes do not regulate EGA onset

As maternally provided chromatin modifiers regulate EGA onset in other model systems, we asked whether this is also the case in *C. elegans* and focused on known modifiers of the germ line - soma distinction. We therefore analyzed whether *vet-4prom*::GFP expression differs in the absence of *mes*

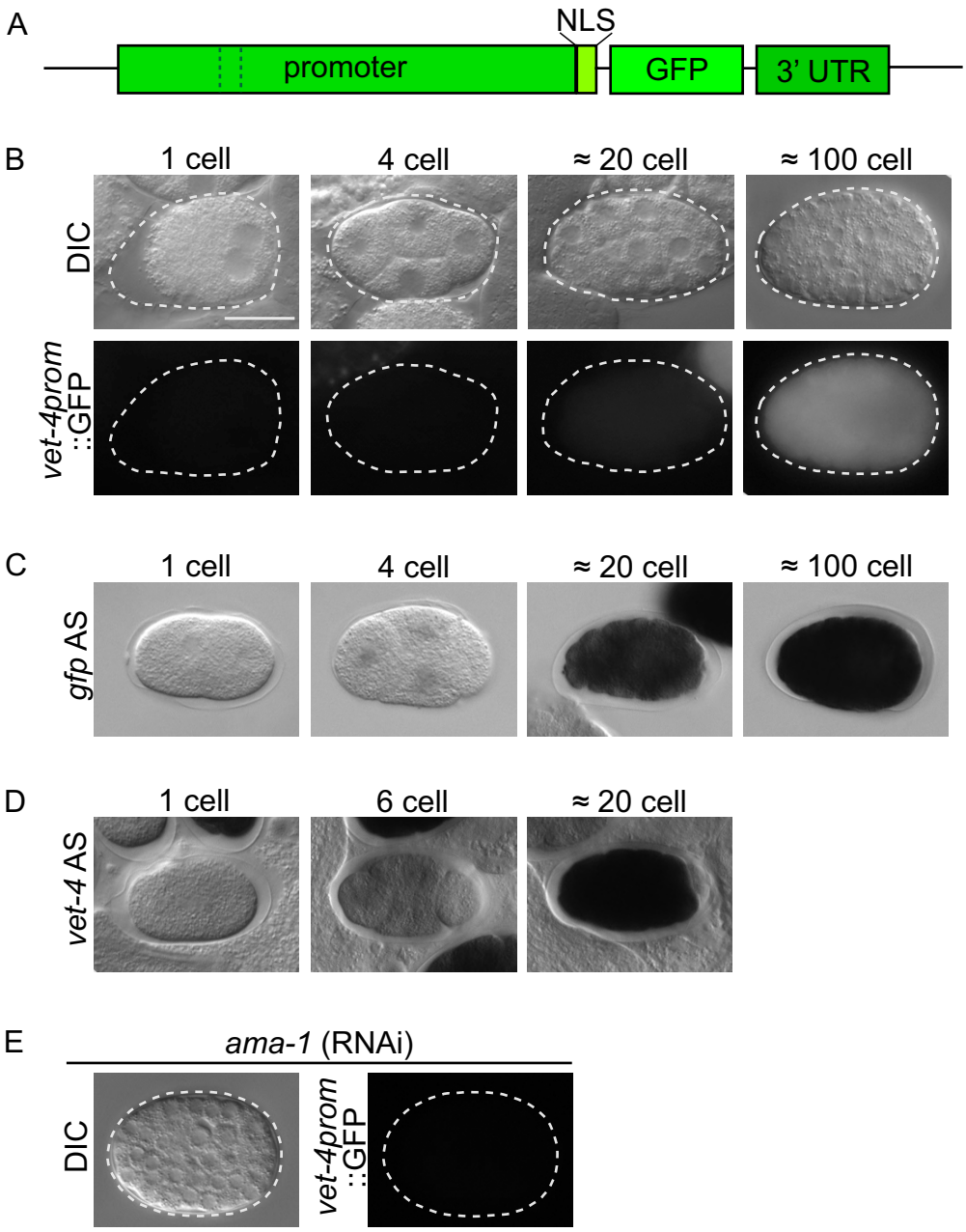


Figure 5: The *vet-4* reporter strain visualizes EGA
(A) The *vet-4* reporter construct - dashed lines indicate point mutations - was bombarded into *unc-119* worms. (B) *vet-4prom*::GFP was not visible before the 20 cell stage. (C) The *vet-4prom::gfp* mRNA was expressed in all somatic cells of the four cell stage embryo but not in the germ line precursor cell. (D) This was in accordance to endogenous *vet-4* mRNA expression. (E) *vet-4prom::GFP* was embryonically expressed and absent in *ama-1* (RNAi) arrested embryos. Scale bar represents 20 µm.

genes and MEC complex members. We either crossed the transgene into mutant worms (*mes-4*, *mes-2*, *let-418*) or used RNAi (*mep-1, hda-1, mes-3, mes-6*) (Figure 6B and data not shown). The *mes* mutants are maternal effect sterile and develop a functional germ line if the gene is maternally expressed. Homozygous mutants derived from homozygous mutant hermaphrodites (M-Z-, for maternal homozygous mutant and zygotic homozygous mutant), however, display germ line defects and develop into sterile adults. For analyzing the GFP expression pattern we therefore always used M-Z- embryos.

If the genes positively regulate EGA onset, we expected a delay in GFP expression in their absence, whereas negative regulation should result in earlier detection of GFP. None of the *mes* mutants showed any difference of the *vet-4prom*::GFP expression compared to the control (Figure 6B and data not shown). From all analyzed MEC complex genes, *mep-1* displayed a severely different GFP expression pattern (Figure 6B and data not shown). As in mock RNAi embryos, GFP expression in *mep-1* (RNAi) embryos started at around the 20 cell stage. However, afterwards they failed to strongly express GFP in all embryonic cells and instead expressed GFP in varying patterns at around the 60 cell stage (Figure 6B). 63 of 67 *mep-1* (RNAi) embryos expressed GFP differently from control embryos, whereas none (1 for *mes-4*) showed different GFP expression for all further quantified mutants (Figure 6C).

The GFP expression differences for *mep-1* (RNAi) embryos were unlikely to result through the MEC complex as we would expect that the other components also show different GFP expression. MEP-1 not only functions in the MEC complex but also interacts with splicing factor related MOG genes, notch co-repressor complex component CIR-1 and transcriptional repressor sumoylated LIN-1 (Belfiore et al., 2002, Kasturi et al., 2010, Miley et al., 2004). However, we first analyzed whether MEP-1 generally affects embryonic gene expression or whether only *vet-4prom*::GFP expression is altered. *mep-1* (RNAi) on PES-10::GFP expressing embryos did not lead to major expression pattern differences (Figure 6D). And *in situ* hybridization against endogenous *vet-4* mRNA did not lead to the expected differences as

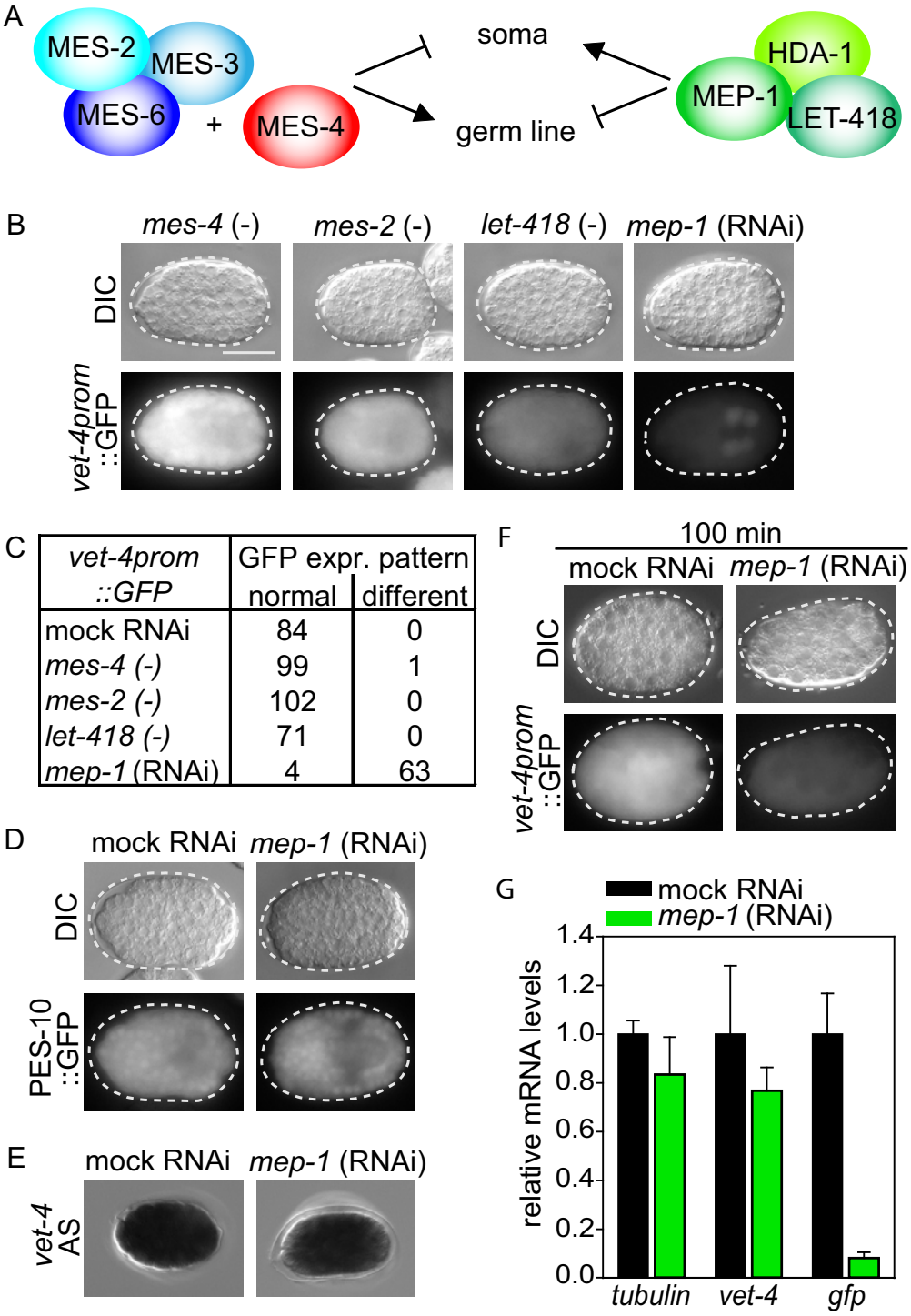


Figure 6: Soma-germ line modifiers do not regulate the onset of EGA
(A) The *mes* genes counteract the MEC complex in maintaining the soma - germ line distinction. (B + C) *vet-4prom*::GFP expression was wild type like in all modifier mutants except for *mep-1* (RNAi) embryos. (D) However, PES-10::GFP expression and (E) endogenous *vet-4* mRNA levels were not effected in *mep-1* (RNAi) embryos. (F) Further, although *vet-4prom*::GFP expression and (G) *gfp* mRNA levels were strongly reduced in 100 min old embryos (0 min = two cell stage), the endogenous *vet-4* mRNA was still highly expressed, indicative for a discrepancy between endogenous and transgenic *vet-4* expression. Scale bar represents 20 µm.

seen for the *vet-4prom*::GFP (Figure 6E). These findings suggest that the *gfp* mRNA might be translational repressed or that there is a discrepancy between endogenous *vet-4* and transgenic *vet-4prom::gfp* transcripts. To discriminate between these two possibilities, we dissected two cell stage embryos from *mep-1* and mock (RNAi) treated hermaphrodites and let them develop for 100 min at room temperature. At this age, GFP intensity is higher in the mock treated embryos than for *mep-1* (RNAi) embryos (Figure 6F). We extracted mRNA of these embryos and analyzed expression levels of endogenous *vet-4* and transgenic *gfp* mRNAs. The control mRNA *tbb-2* was detected at slightly reduced levels in the *mep-1* (RNAi) treated embryos compared to mock RNAi (Figure 6G). The endogenous *vet-4* transcript also only showed minor expression level differences in both mock and *mep-1* (RNAi) embryos (Figure 6G). In contrast, the expression level of the transgenic *gfp* mRNA was severely reduced in *mep-1* (RNAi) embryos compared to mock RNAi embryos (Figure 6G). These findings indicate that endogenous *vet-4* mRNA expression is not as severely affected as expected based on the *vet-4prom*::GFP expression levels. Further, the lower GFP protein levels at this age result from lower *gfp* transcript abundance and not from translational repression of the *gfp* mRNA. In the case of *mep-1* (RNAi), *vet-4prom*::GFP expression is therefore not a direct readout for endogenous *vet-4* mRNA levels. We conclude that the observed GFP expression difference in *mep-1* (RNAi) embryos is a direct consequence of the integrated *vet-4prom::gfp* transgene. These findings demonstrate that the chromatin modifiers of the germ line - soma distinction have no additional role in regulating EGA onset. The *vet-4* reporter strain is a good tool to visualize EGA in the wild type background but careful analyses are needed to verify GFP expression results under RNAi conditions and in mutants.

3.2. Teratomatous cells show early embryonic-like features and differentiate

The *C. elegans* germ line teratoma forms in the adult gonad after depletion of the RNA binding protein GLD-1 and is enhanced if the RNA binding protein MEX-3 is additionally depleted (Ciosk et al., 2006). Germ cells enter meiosis but prematurely re-enter mitosis, express early embryonic genes and differentiate into muscles, neurons and gut cells (Biedermann et al., 2009, Ciosk et al., 2006) (Figure 7A). Re-entry into mitosis and subsequent teratoma formation depends on Cyclin E/*cye-1* which is a direct GLD-1 target and thus ectopically expressed in *gld-1* mutant worms (Biedermann et al., 2009). As teratomas express early embryonic genes and differentiate into somatic tissue, we ask whether ectopic embryonic-like development is a general feature of teratoma formation. We focused on two teratoma strains which both form as a consequence of *gld-1* depletion but differ in their intensity to form a teratoma. *gld-1; fem-1* teratomas develop slowly and due to the *fem-1* mutation, ectopic somatic development can not occur from abnormal fertilization (Biedermann et al., 2009). *gld-1; mex-3* teratomas develop faster than *gld-1* teratomas which might be due to the immediate release of transcriptional repression of GLD-1 and MEX-3 shared target genes (Ciosk et al., 2006).

Transcriptional remodeling precedes EGA in the teratoma

During early embryonic development, first signs of embryonic gene activation mark the resumption of active transcription. Transcriptional inhibition is established during gametogenesis and remains during initial embryogenesis. Although teratomatous cells prematurely re-enter mitosis and thus do not undergo gametogenesis, it is unclear whether embryonic gene activation in the teratoma follows wild type-like transcriptional remodeling. To address this question, we performed immunostaining experiments against the phosphorylated serine residue 5 of the polymerase II CTD subunit (P-Ser5) - a sign for active transcription. In *gld-1; fem-1* mutants, the majority of cells in the

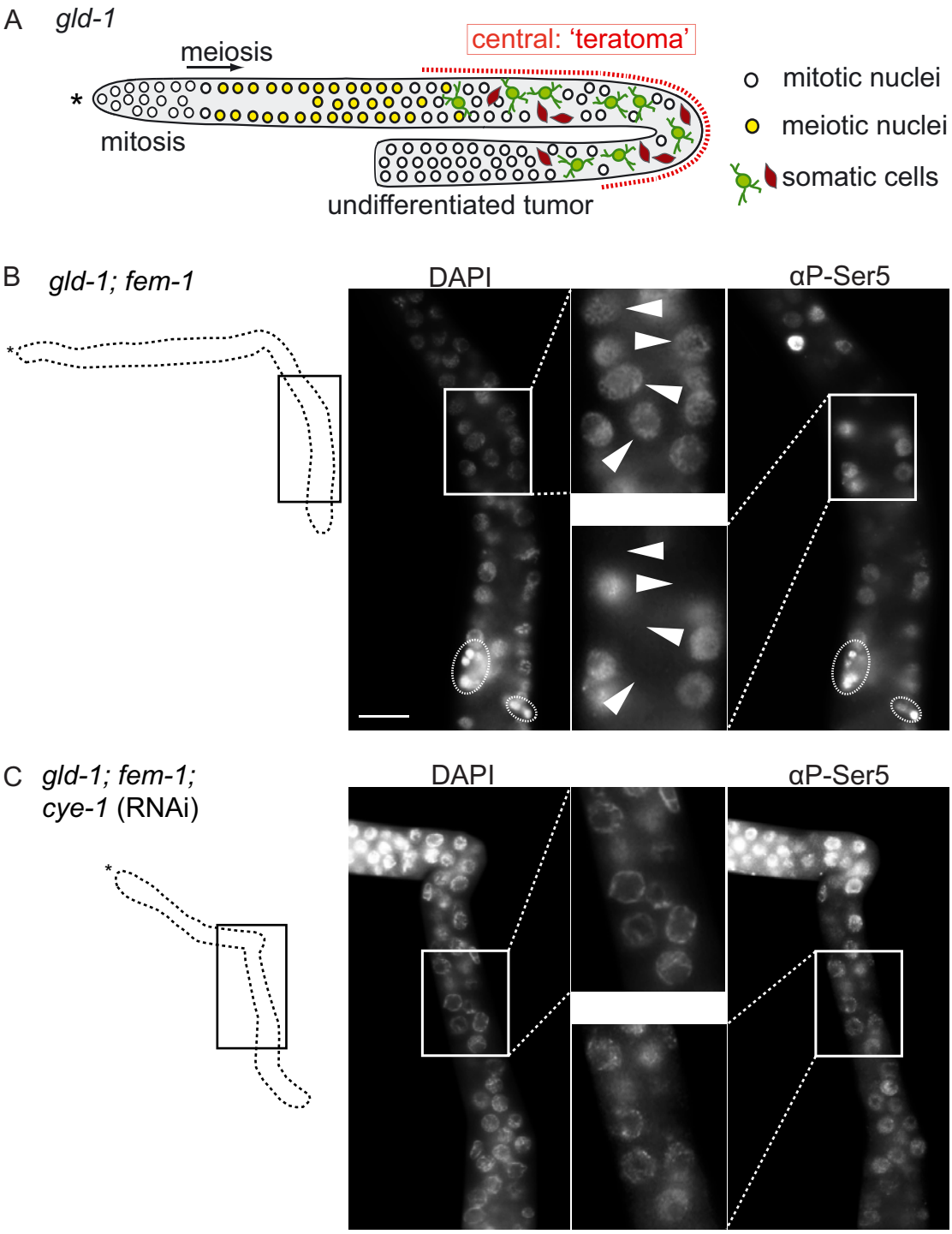


Figure 7: Transcriptional remodeling precedes teratoma formation
(A) A teratoma develops when germ cells re-enter mitosis in the central germ line and differentiate into somatic cells (adapted from Biedermann et al., 2009). (B) The majority of cells in the central germ line carried the P-Ser5 mark and was transcriptionally active as well as cells which re-entered mitosis (encircled). A third population (arrow heads) of cells did not carry the active mark and were transcriptionally silent. (C) This cell population vanished when teratoma formation was inhibited by *cye-1* (RNAi). Scale bar represents 20 µm, asterisks mark distal germ lines.

central part of the gonad carried the P-Ser5 mark and was thus transcriptionally active (Figure 7B). Cells which re-entered mitosis were also transcriptionally active (Figure 7B). Next to transcriptionally active cells, we detected a third population of transcriptional silent cells which lacked the P-Ser5 signal (Figure 7B). Not counting mitotically dividing cell clusters, 35 % of cells in the central part of a *gld-1; fem-1* mutant accounted to this third population. If these cells were indeed undergoing transcriptional remodeling prior to embryonic gene activation, they should not occur if teratoma formation is inhibited. To test this, we repeated the immunofluorescence experiments in *gld-1; fem-1* mutants treated with *cye-1* (RNAi). In these gonads, most nuclei in the central gonad were transcriptionally active and only a total of 3 % did not show a P-Ser5 signal (Figure 7C). These results suggest that wild type like transcriptional remodeling precedes embryonic gene activation in the teratoma.

Early embryonic genes are expressed in two different teratoma backgrounds

Early embryonic transcripts refer to genes which are expressed in somatic blastomeres of the four cell stage embryo. It has been previously shown that early embryonic transcripts are expressed in the *gld-1; fem-1* teratoma (Biedermann et al., 2009). We further expanded this study and asked whether embryonic genes are expressed in both, *gld-1; fem-1* and *gld-1; mex-3*, teratoma lines. By analyzing RNA extracted from wild type and mutant gonads, we found that the early embryonic transcripts *vet-4* and *vet-6* are expressed in both teratoma lines, whereas *vet-1* expression seemed to be restricted to *gld-1; fem-1* mutants and *pes-10* expression occurred only in the *gld-1; mex-3* teratoma (Figure 8A). Note that mRNA levels were normalized to wild type gonads in which embryonic genes are not expressed, yet the highly sensitive RealTime PCR assay detected them as a background noise and relative mRNA levels are thus arbitrary. The *vet-4* mRNA was expressed from teratoma cells as has been shown previously by *in situ* hybridization for *gld-1; fem-1* (Biedermann et al., 2009). Also in *gld-1; mex-3* teratomatous cells, *vet-4* mRNA was strongly expressed (Figure 8B). We further crossed the *vet-*

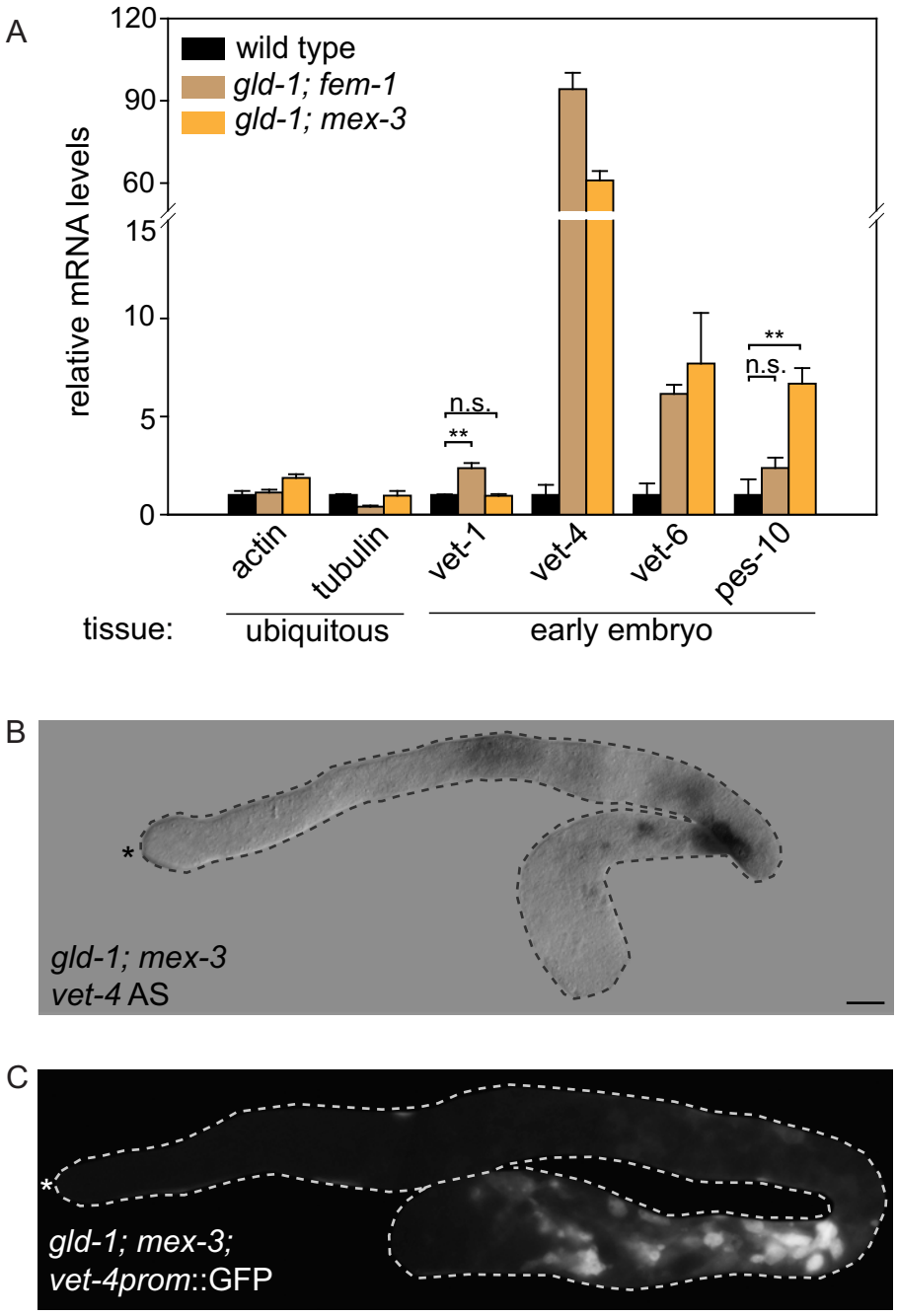


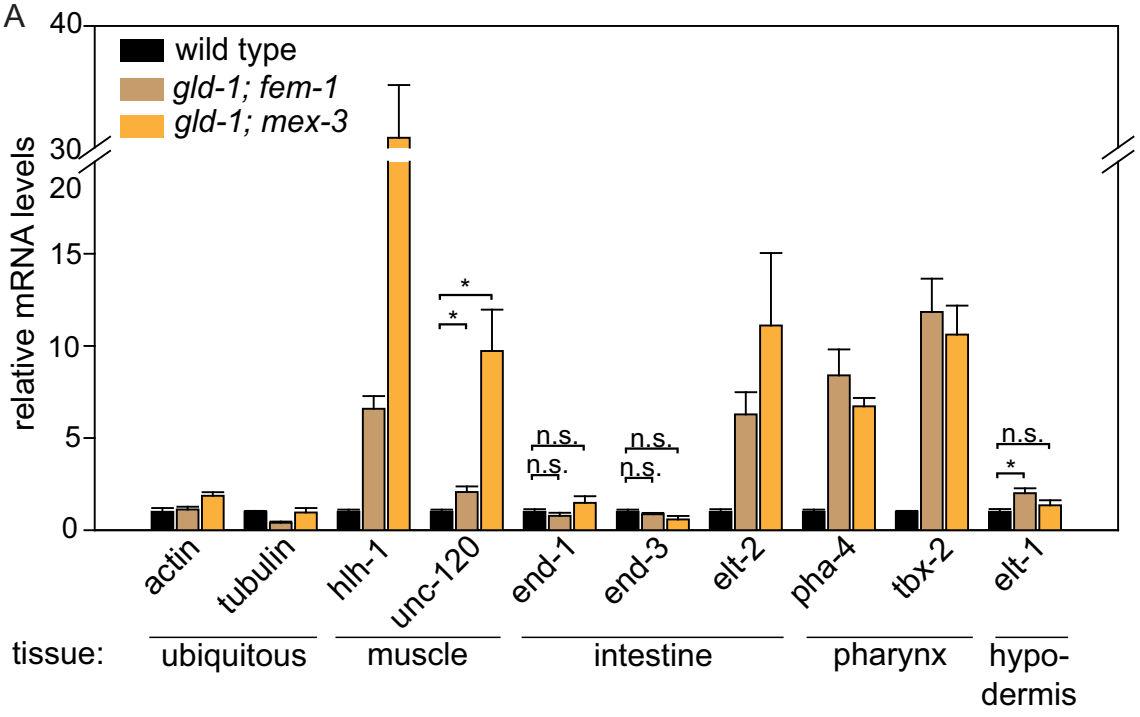
Figure 8: Early embryonic genes are expressed in teratomas
(A) The early embryonic genes *vet-4* and *vet-6* were expressed in two analyzed teratomas whereas *vet-1* seemed to be restricted to the *gld-1; fem-1* and *pes-10* to the *gld-1; mex-3* teratoma (** p<0.01). (B) *vet-4* transcripts were expressed in the central part of the teratomatous *gld-1; mex-3* germ line. (C) High *vet-4prom*::GFP levels were visible in the *gld-1; mex-3* teratoma. (B + C) Scale bar represents 20 µm, asterisks mark distal germ lines.

4prom::gfp reporter strain into the *gld-1 mex-3* mutant background. GFP expression was strong in the central and proximal gonad of *gld-1; mex-3* mutants, indicating functional translation of early embryonic-like mRNAs (Figure 8C).

Teratomatous cells differentiate but show a bias towards neuronal, muscle and pharyngeal tissues

In wild type embryos, lineage specification is initiated shortly after EGA onset. The four cell stage embryo consist of the germ line precursor P2 cell, the ABa cell, the ABp cell and the EMS cell which gives rise to the MS and the gut precursor E cell. First signs of lineage differentiation are detected at the 2E cell stage (after the E cell divided into two cells) in which the gut determinant ELT-2 is expressed.

Previous studies identified high amounts of neuronal and muscle cells as part of the germ line teratoma (Biedermann et al., 2009, Ciosk et al., 2006). To examine it further, we extended the mRNA expression studies to genes which are essential for gut, pharyngeal and hypodermal development. We analyzed genes which are expressed in the embryo at early stages of their lineage specification. As a control of known teratomatously expressed tissues, we analyzed the muscle genes *hlh-1* and *unc-120* and detected both mRNAs in the *gld-1; mex-3* teratoma. *hlh-1* is further highly expressed in the *gld-1; fem-1* teratoma, whereas *unc-120* is detected only little above background levels (Figure 9A). A previous study showed that some *gld-1; mex-3* gonads contained cells with gut characteristics (Ciosk et al., 2006). In our analysis, we found that among the tested gut genes, neither *end-1* nor *end-3* were expressed in the teratoma, whereas the gene *elt-2* was expressed in both teratoma lines (Figure 9A). This finding might be partially explained by the fact that both *end-1* and *end-3* are only transiently expressed in wild type embryos whereas *elt-2* is permanently expressed in gut cells. To further analyze how frequently gut development occurs in the teratoma, we stained for ELT-2 in the *gld-1; mex-3* mutant. According to the previous study, we found some gonads (5 of 50) which had a limited number of 1-2 ELT-2 positive cells (Figure 9B). These results indicate that the teratoma cells are able to



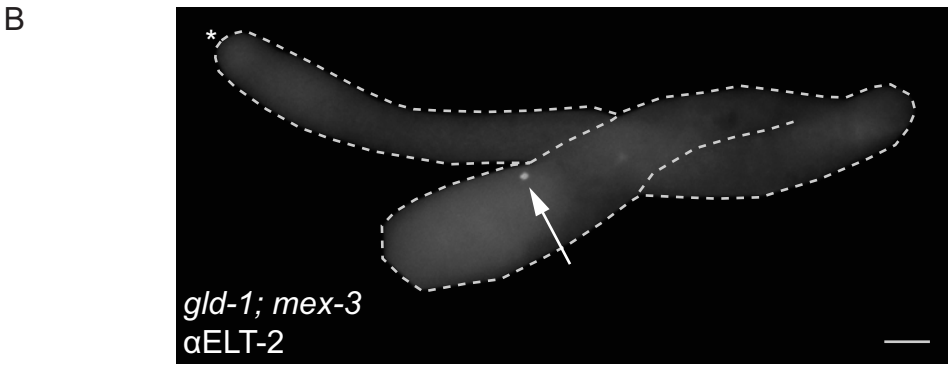


Figure 9: Tissue specific genes are expressed in teratomas
(A) Genes regulating muscle and pharyngeal tissue differentiation were highly expressed in two teratomas whereas a gene involved in hypodermal differentiation was only mildly upregulated in the *gld-1; fem-1* teratoma. Of the analyzed gut differentiation genes, *elt-2* was highly expressed (* $p<0.05$). (B) Only few cells of the teratoma differentiated into gut cells and expressed the ELT-2 protein. Scale bar represents 20 µm, asterisk marks distal germ lines.

differentiate into gut cells, but this pathway is rarely initiated. We additionally analyzed genes which drive pharyngeal differentiation, and detected the genes *pha-4* and *tbx-2* to be highly expressed in both teratoma lines (Figure 9A). We conclude that pharyngeal differentiation takes place at similar frequency in both teratoma lines. Further, we analyzed whether hypodermal genes are expressed in the teratoma and found that the *elt-1* mRNA is only mildly detected above background level (Figure 9A). We conclude that hypodermal differentiation, if at all, only occurs at a very low frequency.
In summary, teratomatous cells differentiate but show a bias towards neuronal, muscle and pharyngeal tissues whereas intestinal and hypodermal tissues are formed at lower frequencies.

Teratomatous cells are developmentally plastic

In *C. elegans*, embryonic lineage specification is mainly due to spatially and temporally restricted expression of cell fate specific transcription factors. Ectopically expressing a cell fate driving transcription factor in the early embryo is sufficient to induce a “cell fate switch” within the entire embryo and thus demonstrates the enormous developmental plasticity of embryonic cells (Fukushige et al., 1998, Fukushige and Krause, 2005). We used this technique to test whether the teratomatous cells are as plastic as wild type embryonic cells. We chose to ectopically express the gut differentiation factor ELT-2 as the gut fate itself is rarely initiated in the teratoma and ectopic gut differentiation should be easily detectable (Figure 9A and B). In wild type embryos, ELT-2 expression is initiated in the 2E gut precursor cells and maintained in all gut cells throughout embryonic, larvae, and adult gut development, presumably through auto-activation. ELT-2 has a lot of target genes throughout all stages of gut development and is believed to be the predominant transcription factor regulating the differentiation and function of the gut (McGhee et al., 2009). One of its target genes is the intermediate filament IFB-2 which is initially expressed during mid-embryogenesis. Additional ELT-2 target genes are *asp-1, haf-9, clec-85, cpr-1* and *sbp-1* (Figure 10A). Ectopic expression of ELT-2 is achieved through heat shocking worms which carry a heat shock promoter driven *elt-2* construct (*hsp::elt-2*)

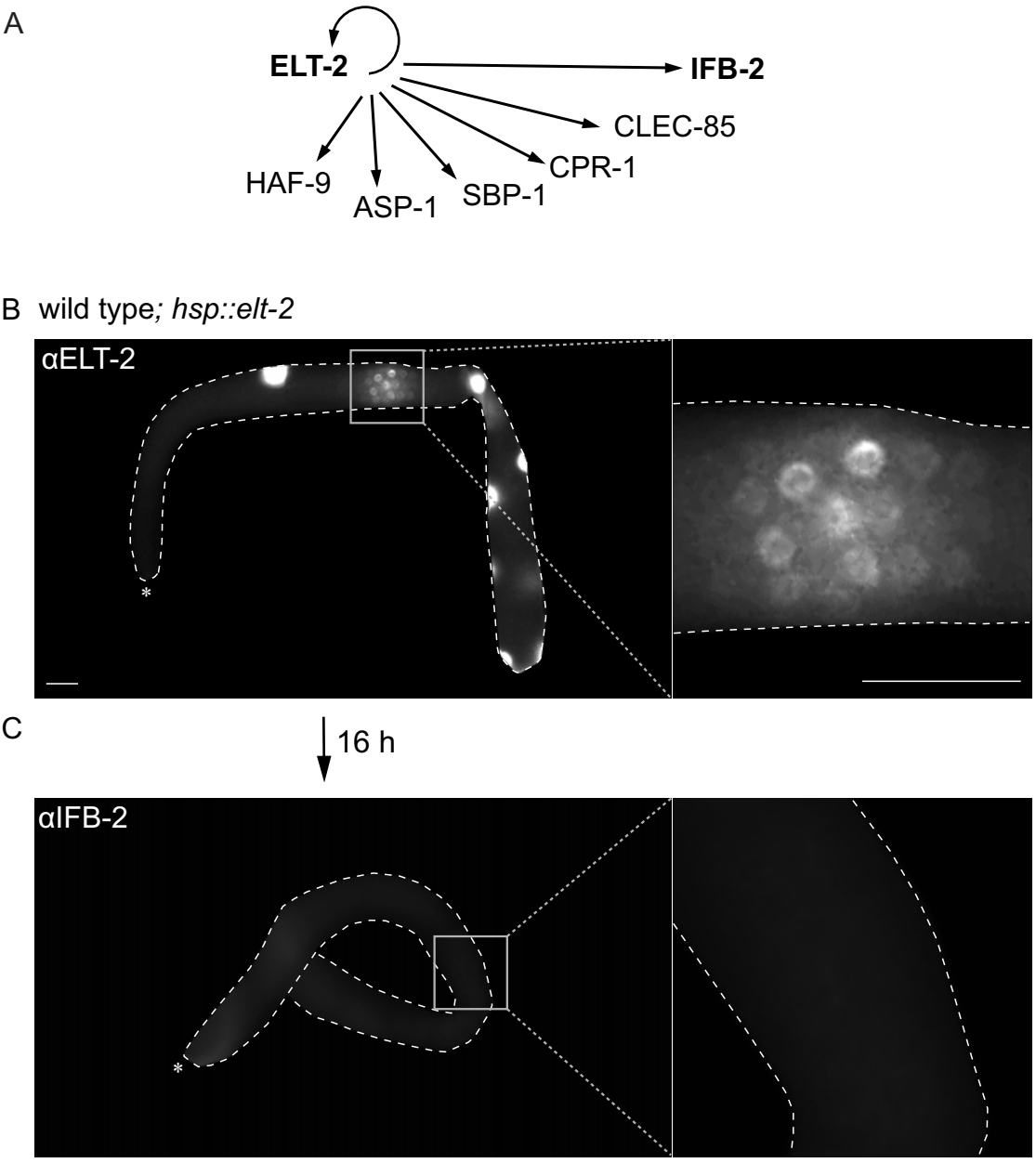


Figure 10: The wild type germ line does not respond to an induced cell fate switch
(A) ELT-2 directly activates a variety of target genes throughout the development of the gut and is known to auto-activate its own expression. (B) ELT-2 was ectopically expressed by a heat shock promoter in an otherwise wild type germ line. 1 h after the heat shock, ELT-2 expression was limited to a few cells in the central part of the gonad. (C) 16 h after the heat shock, the terminal gut differentiation marker IFB-2 was not detectable in the wild type germ line. (B + C) Scale bars represent 20 µm, asterisks mark distal germ lines.

(Fukushige et al., 1998). If ELT-2 is ectopically expressed in embryos, embryonic development arrests and ELT-2 and IFB-2 protein levels are detected in all embryonic cells (Fukushige et al., 1998). We heat shocked *hsp::elt-2* worms for 1 h at 33 °C and analyzed whether wild type germ cells are able to express gut specific genes. In wild type *hsp::elt-2* gonads, we detected weak ELT-2 protein expression, which was restricted to a few cells of the central gonad (Figure 10B). After 16 hours recovery, no IFB-2 signal was detected in the wild type gonad (Figure 10C). We next crossed the *hsp::elt-2* construct into the *gld-1; mex-3* teratoma. If teratomatous cells are as plastic as wild type embryos, we expected strong ELT-2 and IFB-2 expression after recovery from heat shock. In the teratoma, ELT-2 protein was strongly expressed starting 1 h after heat shock (Figure 11A). Further, 16 h after heat shock, the IFB-2 signal was strongly detected in the teratoma (Figure 11B). After 16 h recovery, both *elt-2* and *ifb-2* mRNAs were highly expressed in the *gld-1; mex-3; hsp::elt-2* teratomatous gonad compared to the *gld-1; mex-3* gonad (Figure 11C). Next to *ifb-2*, several other ELT-2 target genes were detected at elevated levels in the *gld-1; mex-3; hsp::elt-2* gonad compared to the *gld-1; mex-3* gonad (Figure 11C). The induced gut differentiation also led to IFB-2 expression in the *gld-1* single mutant teratoma (data not shown). These results strongly suggest that teratomatous cells are plastic and - like wild type embryonic cells - able to adopt various cell fates.

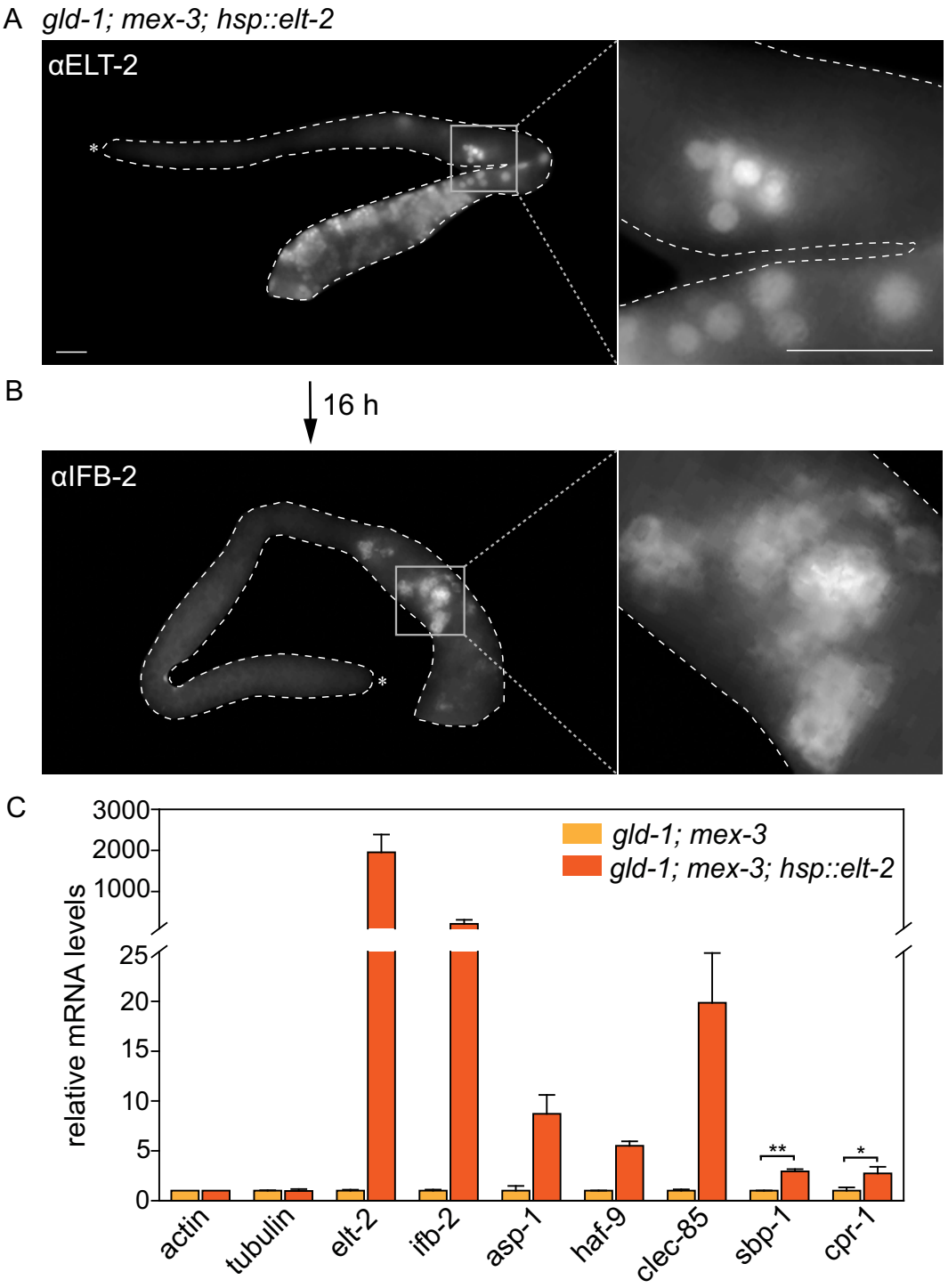


Figure 11: Teratomatous cells respond to an induced cell fate switch
ELT-2 was ectopically expressed by a heat shock promoter in the teratomatous germ line. (A) 1 h after heat shock, the ELT-2 protein was expressed throughout the central and proximal teratomatous gonad. (B) 16 h after heat shock, the terminal gut differentiation marker IFB-2 was strongly expressed in the central and proximal teratomatous gonad. (A + B) Scale bars represent 20 µm, asterisks mark distal germ lines. (C) Next to IFB-2, all analyzed ELT-2 target genes were highly upregulated in the *gld-1; mex-3; hsp::elt-2* germ line after 16 h recovery from heat shock (* $p<0.05$, ** $p<0.01$).

3.3. Ovulated but unfertilized *C. elegans* oocytes show early embryonic-like features but do not differentiate

In most sperm-defective mutants, oocytes are ovulated but not fertilized as the sperm fails to enter the oocyte. These ovulated oocytes are pushed into the uterus of the worm, and initiate embryogenesis as they progress through an abnormal meiosis and undergo endoreplication (McNally and McNally, 2005, Ward and Carrel, 1979). We analyzed *fem-1* mutants which are unable to produce sperm. As oocytes depend on a sperm derive signal for ovulation, oocytes in *fem-1* mutants accumulate in the proximal gonad arm. Some oocytes, however, are able to escape this block and are ovulated. As these oocytes show some features of early embryogenesis, we ask how far abnormal embryonic development goes.

Unfertilized oocytes have somatic characteristics

Early in wild type embryonic development, a combination of asymmetric segregation and active degradation of germ line specific proteins and RNAs establishes a somatic environment in all cells except the germ line precursor cell. Accordingly, maternally provided RNAs are classified into two categories: maternal class I RNAs are distributed equally in all embryonic cells whereas maternal class II RNAs are present only in germ line precursor cells. To test whether ovulated oocytes lose germline and acquire somatic characteristics, we analyzed maternal class I and II mRNA distribution by *in situ* hybridization. The mRNAs *tbb-2* and *dpy-30* belong to ubiquitously distributed maternal class I RNAs and were detected at equal levels in *fem-1* mutant gonads, as well as in ovulated *fem-1* oocytes (Figure 12A and B). The maternal class II RNA *pos-1* was strongly expressed in the *fem-1* mutant gonad (Figure 12C). However, *pos-1* was absent from *fem-1* ovulated oocytes (Figure 12C). The same results occurred with the maternal class II mRNA *cey-2* (Figure 12D).
Further, germ line specific proteins are known to be absent from somatic cells. For example, the protein PIE-1 accumulates in the proximal gonad arm but is restricted to the germ line precursor cells in the embryo. We stained for PIE-1 protein and found it to be present in the wild type and *fem-1* mutant proximal

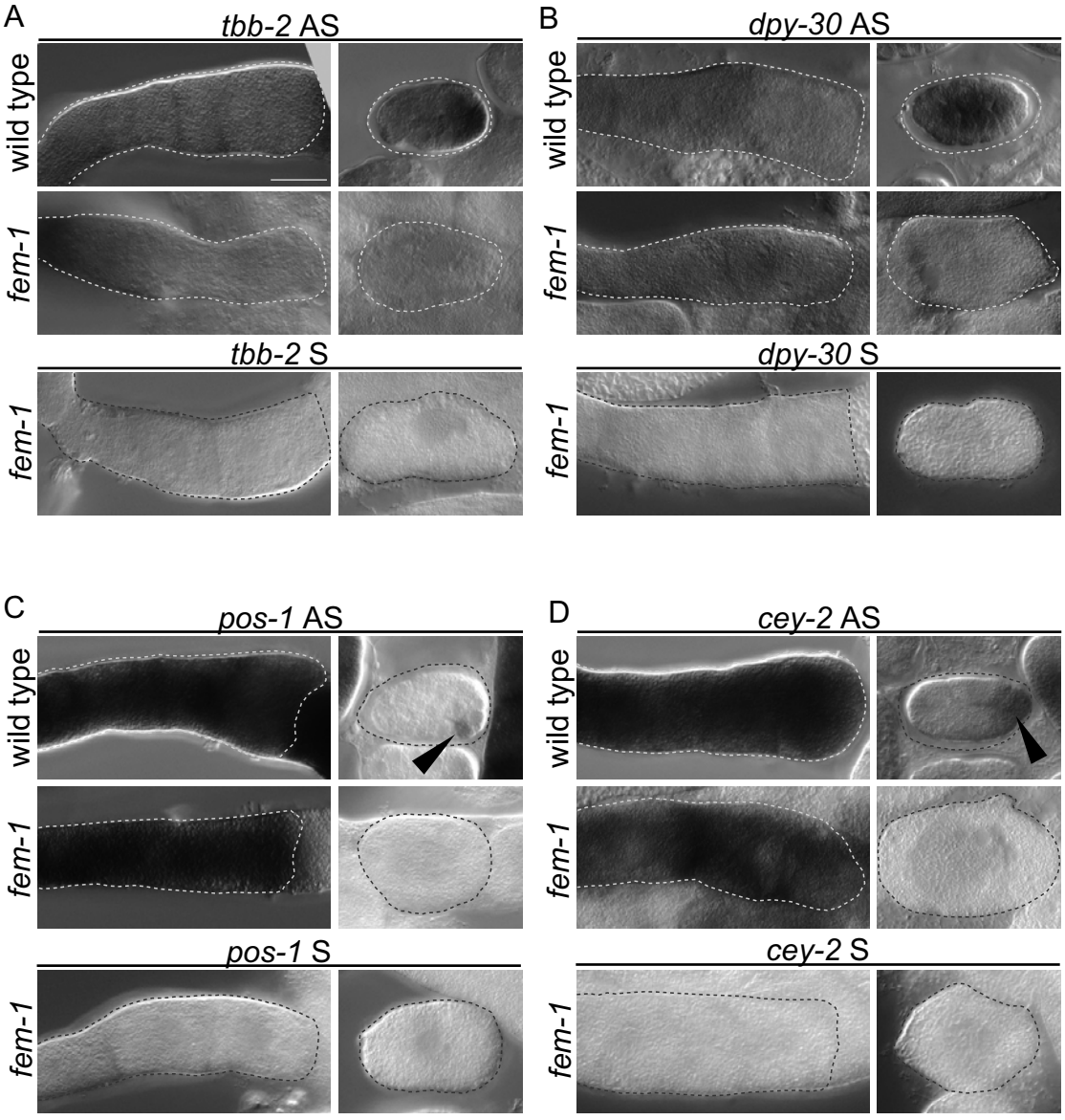


Figure 12: Unfertilized oocytes express maternal class I mRNAs but not maternal class II mRNAs

(A + B) The maternal class I mRNAs *tbb-2* and *dpy-30* were uniformly expressed in the wild type and *fem-1* gonad (left) as well as in wild type embryos and unfertilized oocytes (right). (C + D) The maternal class II mRNAs *pos-1* and *cey-2* were uniformly expressed in the wild type and *fem-1* gonad (left) and present in the wild type embryonic germ line precursor cell (right, arrowhead). Both transcripts were absent from all somatic cells of the wild type embryo and from unfertilized oocytes (right). Scale bar represents 20 µm.

gonad (Figure 13A and B). In wild type embryos, PIE-1 localized exclusively to the germ line precursor cell (Figure 13 A), but was not detected in ovulated *fem-1* oocytes (Figure 13B).

Together, these results show that ovulated but unfertilized oocytes are capable of embryonic-like mRNA and protein degradation.

Unfertilized oocytes are transcriptionally active

In wild type embryos, somatic cells are transcriptionally active beginning at the four cell stage. Transcriptional onset depends on the degradation of OMA-1/2 proteins which sequester the general transcription factor TAF-4 to the cytoplasm. We asked, whether ovulated oocytes which acquire somatic characteristics also start transcribing their genome.

OMA-1::GFP expressing worms were exposed to *fem-1* RNAi. GFP levels were high in the proximal gonad arms and in newly ovulated oocytes. However, unfertilized oocytes gradually lost GFP expression which indicates functional OMA-1/2 degradation and the release of TAF-4 (Figure 14A).

Staining against P-Ser5, a marker for transcriptional initiation, revealed that oocytes located in the proximal gonad arm were transcriptionally silent (Figure 14B). Ovulated oocytes which started endoreplicating were transcriptionally active as they showed a strong signal for P-Ser5 (Figure 14B). Further, staining against P-Ser2, a marker for transcriptional elongation, confirmed these findings (Figure 14C).

Thus, unfertilized oocytes reinitiate transcription.

Unfertilized oocytes express early embryonic but not lineage specific genes

As unfertilized oocytes have somatic characteristics and started transcription, we were wondering what kind of transcripts they produce. We therefore extracted RNAs from these oocytes and compared it to RNA extracted from transcriptionally silent 1- and 2-cell stage embryos and transcriptionally active mixed stage embryos (early- to mid-embryogenesis). We found that unfertilized oocytes express the early embryonic transcript *vet-4* (Figure 15A).

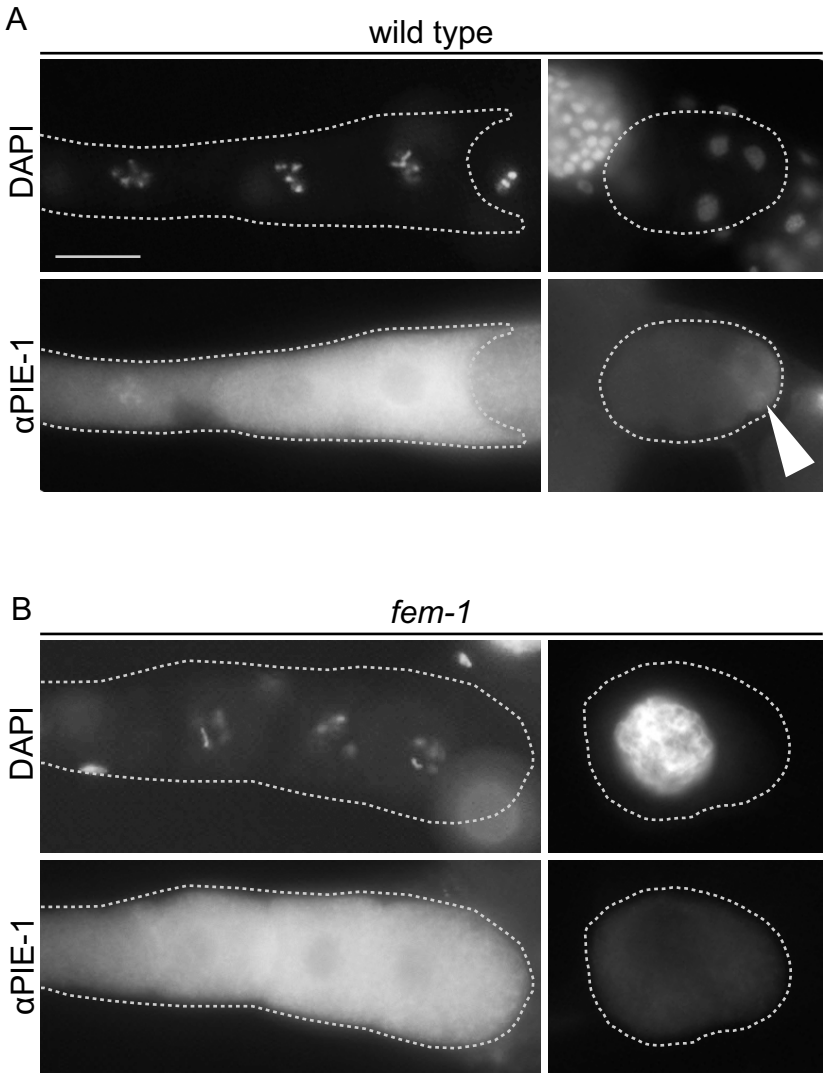


Figure 13: Unfertilized oocytes degrade the germ line specific protein PIE-1

(A) PIE-1 protein was strongly expressed in the wild type gonad but absent from the somatic cells of the embryos where it localized only to the germ line precursor cell (arrowhead). (B) In *fem-1* mutants, PIE-1 was strongly expressed in the proximal gonad but not detectable in unfertilized oocytes. Scale bar represents 20 µm.

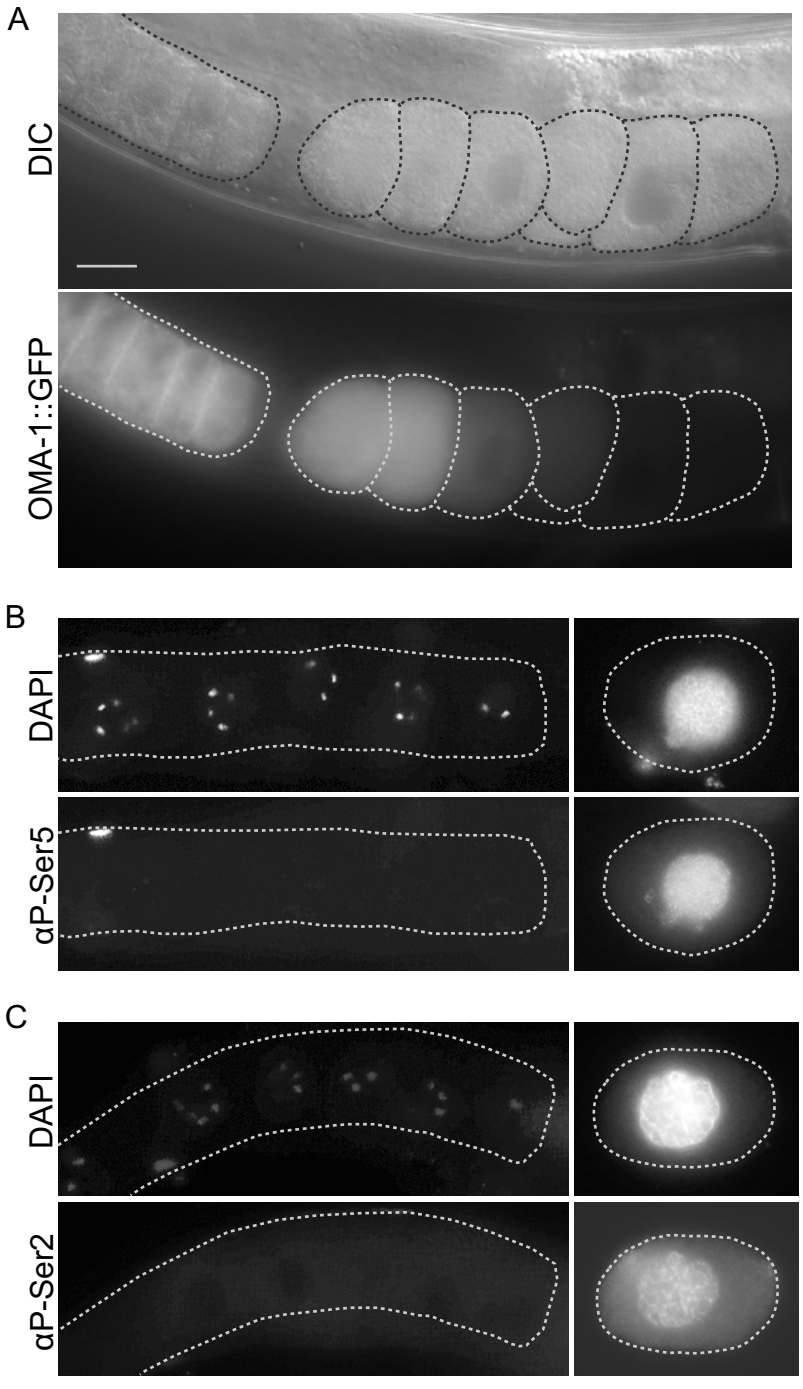


Figure 14: Unfertilized oocyte degrade OMA-1 and are transcriptionally active
(A) After ovulation, unfertilized *fem-1* oocytes degraded the transgenic OMA-1::GFP. (B + C) Oocytes residing in the proximal gonad arm were transcriptionally silent. Ovulated but unfertilized oocytes reinitiated transcription as detected by a marker for (B) transcriptional initiation and (C) transcriptional elongation. Scale bar represents 20 µm.

vet-4 mRNA was absent from proximal *fem-1* mutant gonads but exclusively expressed in ovulated oocytes as shown by *in situ* hybridization (Figure 15 B). We next asked whether early embryonic transcripts are translated into protein and crossed the *vet-4prom*::GFP reporter into *fem-1* mutants. We detected a robust GFP expression in unfertilized oocytes (Figure 15C) which got stronger as unfertilized oocytes age. The same result was found with a PES-10::GFP reporter strain indicating general EGA onset (Figure 15D). As in wild type embryonic development first signs of lineage specification are detected beginning at the 2E stage, we further analyzed expression levels of embryonic lineage specification markers for muscle, intestinal, pharyngeal and hypodermal development. Surprisingly, we found that none of the tested candidates were expressed as the detected levels were similar to the levels of transcriptionally silent 1- and 2-cell stage wild type embryos (Figure 15A). These findings suggest that unfertilized oocytes initiate EGA but are unable to transcribe lineage specific genes.

Unfertilized oocytes are not developmentally plastic

Do unfertilized oocytes remain in an early embryonic state because they are unable to express lineage initiating transcription factors or because these transcription factors are unable to initiate their specific cell fate? To answer this question, we ectopically expressed the gut initiation factor ELT-2 via the *hsp::elt-2* construct in unfertilized oocytes and analyzed whether these oocytes show any sign of gut differentiation. We were unable to cross the *hsp::elt-2* construct into the *fem-1* temperature sensitive strain but succeeded to obtain ovulated but unfertilized oocytes via two generation *fem-1* RNAi at 20 °C. We dissected unfertilized oocytes from worms, heat shocked, and analyzed oocytes 1 hour and 6 hours after the heat shock.

After 1 hour recovery, *elt-2* mRNA was highly expressed in control embryos carrying the *hsp::elt-2* construct compared to lower endogenous levels of wild type embryos (Figure 16A). Similar, strong *elt-2* mRNA expression was detected in unfertilized oocytes which carried the *hsp::elt-2* construct but no expression was observed in unfertilized oocytes which did not carry the *hsp::elt-2* construct (Figure 16A). At this stage of development, *ifb-2* mRNA

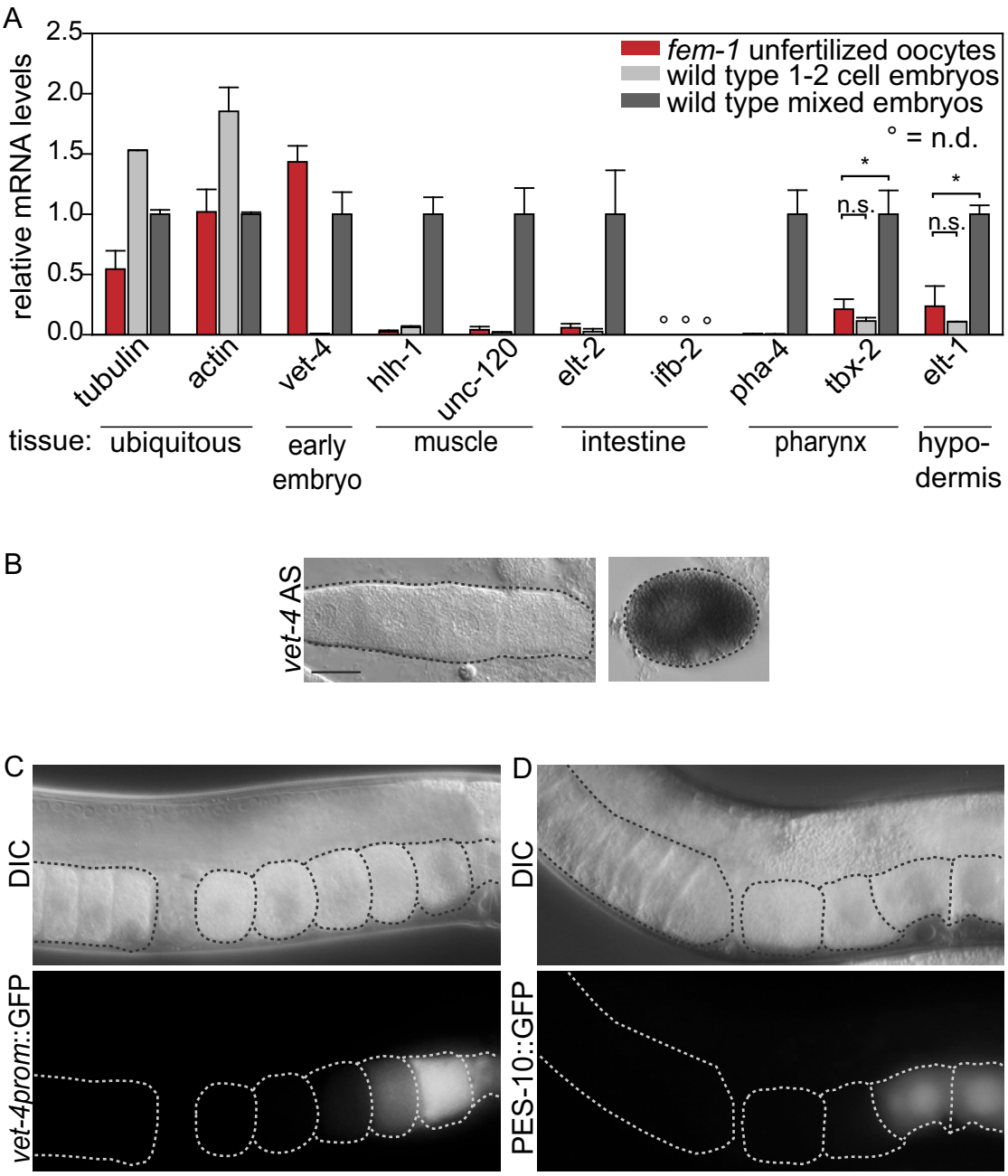


Figure 15: Unfertilized oocytes express early embryonic but no tissue specific genes
(A) Gene expression analysis detected the early embryonic gene *vet-4* to be expressed in unfertilized oocytes. None of the analyzed tissue specific genes was expressed in unfertilized oocytes as they all had similar expression levels as transcriptionally silent 1- and 2-cell stage embryos (* $p<0.05$). (B) *vet-4* transcripts were absent from the *fem-1* germ line but strongly detectable in unfertilized oocytes. (C + D) Reporter strains for the early embryonic genes *vet-4* and *pes-10* were expressing GFP in unfertilized oocytes. Scale bar represents 20 µm.

was not yet detectable (Figure 16A). Wild type embryos which did not carry the *hsp::elt-2* construct expressed ELT-2 protein locally in endogenous gut cells whereas *hsp::elt-2* embryos showed strong ELT-2 expression in every cell of the embryo (Figure 16B). 7 of 16 analyzed unfertilized oocytes which carry the *hsp::elt-2* construct stained positive for the ELT-2 protein, whereas 0 of 15 control oocytes showed ELT-2 protein expression (Figure 16C). These findings indicate a successful ectopic expression of the transcription factor. However, it is unclear whether the ELT-2 negative unfertilized oocytes were not transcribing the *elt-2* RNA after heat shock or were not translating the *elt-2* mRNA.

As unfertilized oocytes have no eggshell and are therefore more fragile than wild type embryos, we shortened the maximal recovery time after heat shock to 6 hours. In *hsp::elt-2* embryos, *elt-2* mRNA levels remained higher than endogenous levels in wild type embryos (Figure 16D). Note that the wild type embryos which do not carry the *hsp::elt-2* construct continue embryonic development including multiplying *elt-2* expressing gut cells, whereas *hsp::elt-2* embryos arrest development shortly after heat shock. As all mRNA levels were normalized to the wild type embryo, the “decrease” in *elt-2* levels in *hsp::elt-2* embryos might just reflect an increase in endogenous *elt-2* mRNA expression. In any case, high *elt-2* mRNA expression in *hsp::elt-2* embryos indicates activation of the gut lineage pathway. 6 hours of recovery time was sufficient to detect equal levels of *ifb-2* mRNA in *hsp::elt-2* and wild type embryos (Figure 16D). IFB-2 protein expression was restricted to endogenous gut cells in developing wild type embryos. In contrast, IFB-2 was uniformly expressed in heat shocked and arrested hsp::elt-2 embryos (Figure 16E). In unfertilized oocytes, *elt-2* mRNA levels were dramatically downregulated to below endogenous embryonic levels (Figure 16D). This finding already indicates that the heat shock induced ELT-2 protein did not activate the expression of endogenous *elt-2*. In agreement, no *ifb-2* mRNA could be detected in unfertilized oocytes which carried the *hsp::elt-2* construct (Figure 16D). Further, 0 of 8 unfertilized *hsp::elt-2* oocytes stained positive for the IFB-2 protein, similar to 0 of 21 control oocytes (Figure 16F).

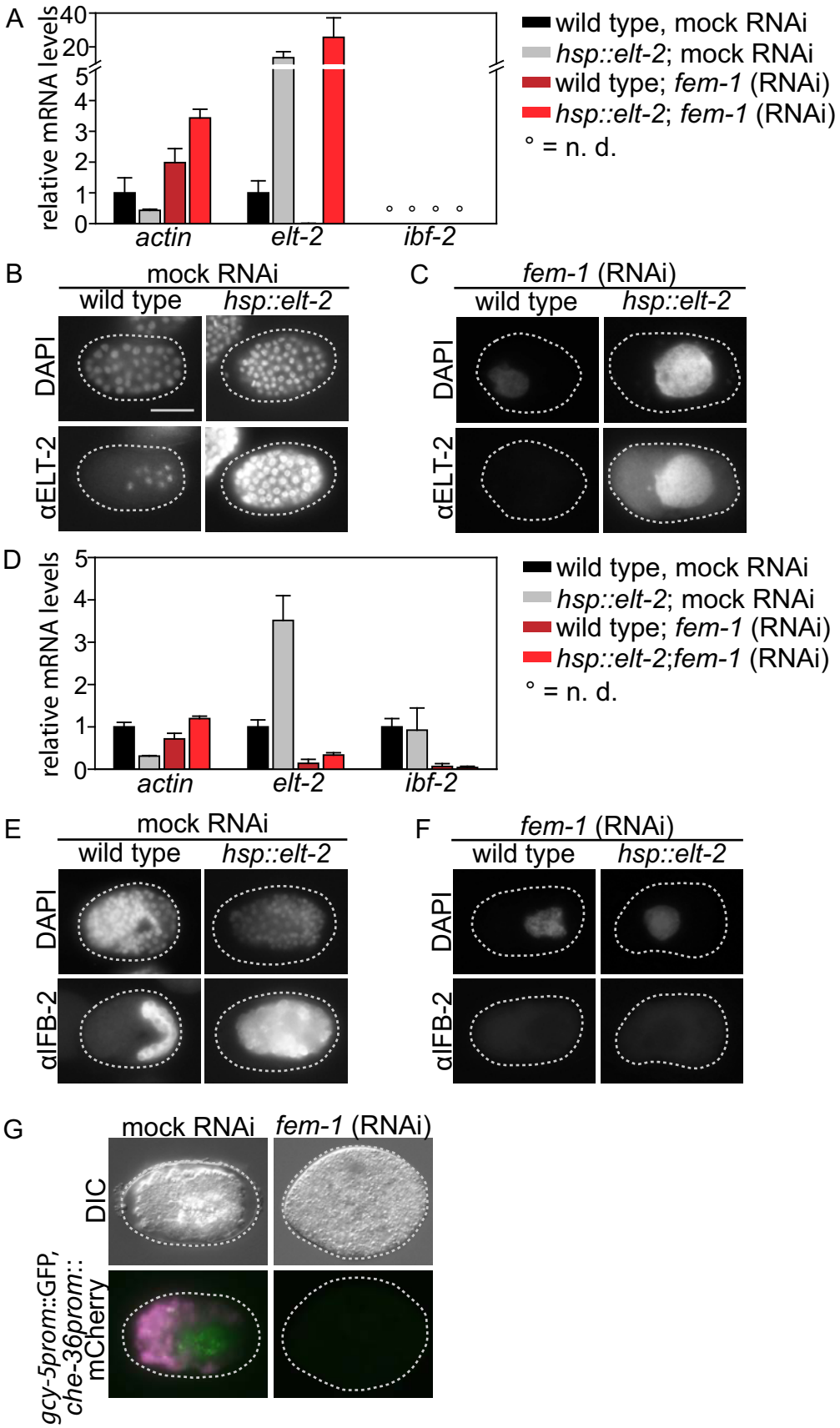


Figure 16: Unfertilized oocytes are unable to respond to an induced cell fate switch

The gut differentiation factor ELT-2 was ectopically expressed via a heat shock

promoter. (A) After 1 h recovery, *elt-2* mRNA was highly expressed in embryos and unfertilized oocytes which carried the *hsp::elt-2* construct compared to control embryos and unfertilized oocytes. (B + C) Both, *hsp::elt-2* embryos and *hsp::elt-2* unfertilized oocytes strongly expressed the ELT-2 protein. Control embryos expressed ELT-2 only in endogenous gut cells and unfertilized control oocytes did not express ELT-2. (D) After 6 h recovery, *elt-2* mRNA was overexpressed in *hsp::elt-2* embryos, but not in unfertilized oocytes. The gut terminal differentiation gene *ifb-2* was only detected in embryos but not in unfertilized oocytes. (E) The IFB-2 protein was detected in endogenous gut cells in the control embryos and uniformly expressed in *hsp::elt-2* embryos. (F) Neither wild type nor *hsp::elt-2* unfertilized oocytes expressed IFB-2 protein. (G) When neuronal differentiation was induced by heat shock driven CHE-1 expression, only embryos expressed the neuronal markers *gcy-5* and *che-36* after 16 h recovery, whereas unfertilized oocytes did not express any neuronal marker. Scale bar represents 20 µm.

To confirm that these findings were not specific for induced gut differentiation, we repeated the heat shock experiment in embryos which expressed the neuronal differentiation factor *che-1* under a heat shock promoter. Neuronal development is visualized by *ceh-36prom::mcherry* and *gcy-5prom::gfp* reporters. Previously, neuronal differentiation upon heat shock has been described in the germ line of *lin-53* depleted worms (Tursun et al., 2010). In mock (RNAi) embryos, heat shock was sufficient to induce strong *ceh-36prom*::mCherry and *gcy-5prom*::GFP expression which indicates neuronal differentiation (Figure 16G). 16 h recovery after heat shock was needed for reporter expression, thus also fragile *fem-1* (RNAi) unfertilized oocytes were analyzed 16 h post heat shock. In unfertilized oocytes, no mCherry or GFP expression was detected (0 of 21 oocytes) which confirmed previous findings for induced gut differentiation.

Taken together, lineage driving transcription factors were not sufficient to drive lineage differentiation in unfertilized oocytes. This suggests that unfertilized oocytes remain in an early embryonic state and they do not reach the wild type like plasticity state.

1-cell stage arrest and impaired meiosis are not causing the differentiation defect of unfertilized oocytes

Unfertilized oocytes are unable to progress through normal meiosis and instead arrest at anaphase I without forming any polar body (McNally and

McNally, 2005). They further do not develop an eggshell and arrest at the 1-cell stage, presumably due to missing sperm derived centrioles and the inability to form a spindle. Both, failure in polar body formation and 1-cell stage arrest, could be a direct consequence of a missing eggshell (reviewed in Johnston and Dennis, 2011). To investigate whether 1-cell stage arrest and impaired meiosis cause the differentiation failure in unfertilized oocytes, we analyzed embryos depleted for essential cytokinesis and meiosis factors. We further modified the induced gut differentiation method by crossing an *ifb-2::cfp* reporter into the *hsp::elt-2* carrying worms (Husken et al., 2008). Heat shock resulted in the visible expression of IFB-2::CFP in mock RNAi treated embryos after 16 h recovery (Figure 17B).

To test whether differentiation requires successful cytokinesis, we repeated the experiments in *zen-4* mutants. ZEN-4 is required for cytokinetic events in the early embryo and *zen-4* mutants fail to undergo cytokinesis, including polar body extrusion, and arrest as multinucleated 1-cell stage embryos (Raich et al., 1998). Embryos derived from *zen-4* (RNAi) exposed *vet-4prom::gfp* reporter worms showed normal levels of GFP expression indicating functional expression of early embryonic gens (data not shown). Further, RNA expression analysis of *zen-4* (RNAi) embryos revealed active transcription of the early embryonic genes *vet-4* and *pes-10*, as well as expression of the lineage specific markers *tbx-2* and *elt-1* (Figure 17A). *zen-4* RNAi treated *hsp::elt-2; ifb-2::cfp* embryos were further able to respond to the induced cell fate switch (10 of 14) (Figure 17B).

To check for the importance of maternal meiosis, we chose to analyze *mei-1* mutant embryos. MEI-1 functions during maternal meiosis leading to an impaired polar body formation and a maternal effect lethal phenotype (Mains et al., 1990). *vet-4* reporter embryos derived from *mei-1* (RNAi) treated hermaphrodites expressed GFP (data not shown). Further, 19 of 28 *mei-1* (RNAi) treated *hsp::elt-2; ifb-2::cfp* embryos were expressing IFB-2::CFP and were thus able to respond to the induced cell fate switch (Figure 17B).

Taking together, cytokinetic and meiotic defects are unlikely to cause the differentiation failure of unfertilized oocytes.

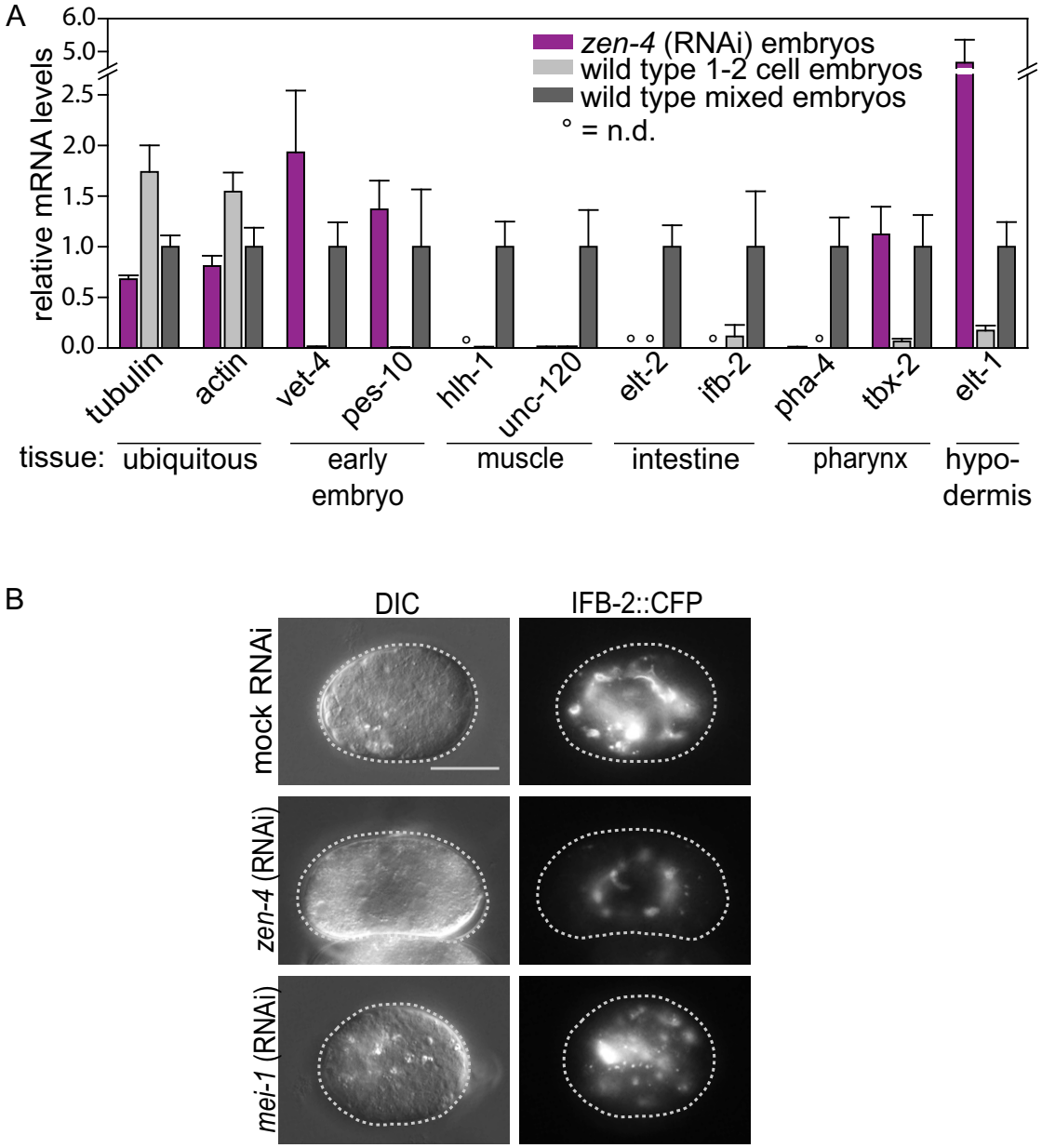


Figure 17: Cytokinesis and meiosis defective embryos differentiate
zen-4 (RNAi) embryos do not undergo cytokinesis, including polar body formation. (A) RNA expression analysis revealed that *zen-4* (RNAi) embryos expressed the early embryonic genes *vet-4* and *pes-10* as well genes regulating the pharyngeal and hypodermal tissue specification. (B) *zen-4* (RNAi) as well as meiosis defective *mei-1* (RNAi) embryos expressed the terminal gut differentiation marker IFB-2::CFP after ectopic ELT-2 expression in *hsp::elt-2; ifb-2::cfp* embryos. Scale bar represents 20 µm.

Sperm inherited components are not leading to developmental plasticity

We next investigated the influence of paternal components on the ability to differentiate. In *C. elegans*, eggshell formation also depends on sperm entry. To discriminate between sperm inherited components, e.g. centrioles, and sperm induced eggshell formation, we chose to analyze *spe-11* mutant embryos. SPE-11 is only inherited through sperm but although associated with sperm chromatin, it is not needed for proper spermatogenesis. (Chu et al., 2006, Browning and Strome, 1996). SPE-11 functions during embryogenesis to establish the polyspermy barrier and to form a functional eggshell (Johnston et al., 2010). *spe-11* embryos do not establish a functional eggshell, fail to undergo cytokinesis and progress through an abnormal meiosis (L'Hernault et al., 1988, McNally and McNally, 2005). However, all sperm provided factors are supposed to be functional in *spe-11* mutant sperm. Embryos coming from *spe-11* mutants expressed the early embryonic genes *vet-4* and *pes-10* but of all analyzed lineage markers, only *elt-1* mRNA was expressed (Figure 18A). *elt-1* mRNA is embryonically expressed and associated with hypodermal development but it has a second function during spermatogenesis and is present in mature sperm (del Castillo-Olivares et al., 2009, Page et al., 1997). Since *spe-11* embryos exhibit strong polyspermy, the origin of the detected *elt-1* mRNA is unclear. However, if *spe-11* embryos are able to differentiate, they should also respond to an induced cell fate switch.

We induced gut differentiation by heat shocking *spe-11; hsp::elt-2* embryos. After 1 h recovery, *elt-2* mRNA was highly expressed in *spe-11; hsp::elt-2* embryos but absent from *spe-11* embryos (Figure 18B). ELT-2 protein was further detected in 13 of 21 *spe-11; hsp::elt-2* embryos, but in 0 of 8 *spe-11* embryos (Figure 18C). This indicates that functional ELT-2 protein was present in 50 % of the *spe-11; hsp::elt-2* embryos.

As spe-11 embryos fail to develop a functional eggshell and are therefore as fragile as unfertilized oocytes, we chose to limit the recovery time to 6 h and rely on IFB-2 staining rather than IFB-2::CFP expression for further analysis. Thus 6 hours after heatshock, *elt-2* mRNA was strongly expressed in *spe-11; hsp::elt-2* embryos but not in *spe-11* embryos (Figure 18D). The strong *elt-2*

expression in transgenic *spe-11* embryos was in contrast to the rapid decrease of *elt-2* mRNA levels in heat shocked *hsp::elt-2* unfertilized oocytes. However, it is unclear whether the high elt-2 mRNA levels were newly synthesized endogenous transcripts or stable remnants of the heat shock. In contrast to high *elt-2* mRNA levels, no *ifb-2* mRNA or IFB-2 protein was detected in wild type and transgenic *spe-11* embryos (wild type: 0 of 38; transgenic: 0 of 31) (Figure 18D and E). These findings indicate that as for unfertilized oocytes, *spe-11* embryos are unable to respond to an induced cell fate switch.

As SPE-11 is associated with sperm DNA it might modify the paternal genome during spermatogenesis and *spe-11* embryos will lack any paternally modified loci which might be necessary for the differentiation ability. We therefore analyzed whether embryos derived from DNA lacking sperm are able to differentiate. 16 of 18 embryos derived from untreated or mock RNAi treated *hsp::elt-2; ifb-2::cfp* hermaphrodites strongly and uniformly expressed IFB-2::CFP (Figure 18F). We next used *fem-1* (RNAi) to feminize worms of this strain and crossed these females with *emb-27* males which were described to lack sperm DNA but otherwise were able to fertilize oocytes (Sadler and Shakes, 2000). Absence of sperm DNA was confirmed by DAPI staining and revealed the loss of DNA in approximately 90 % of sperm (data not shown). 11 of 13 embryos derived from these crosses were able to respond to the induced cell fate switch and expressed IFB-2::CFP (Figure 18F).

The presence of paternally inherited components, like centrioles, does therefore not lead to the ability to differentiate and the embryonic plasticity of wild type embryos.

A functional eggshell might be partially responsible for the ability to differentiate

Although both, unfertilized *fem-1* oocytes and fertilized *spe-11* embryos, arise from completely different backgrounds, namely maternal only and biparental, they share the inability to differentiate. Yet, they are phenotypically similar as they fail to develop a functional eggshell, undergo an impaired maternal meiosis leading to the absence of polar bodies, and are unable to progress

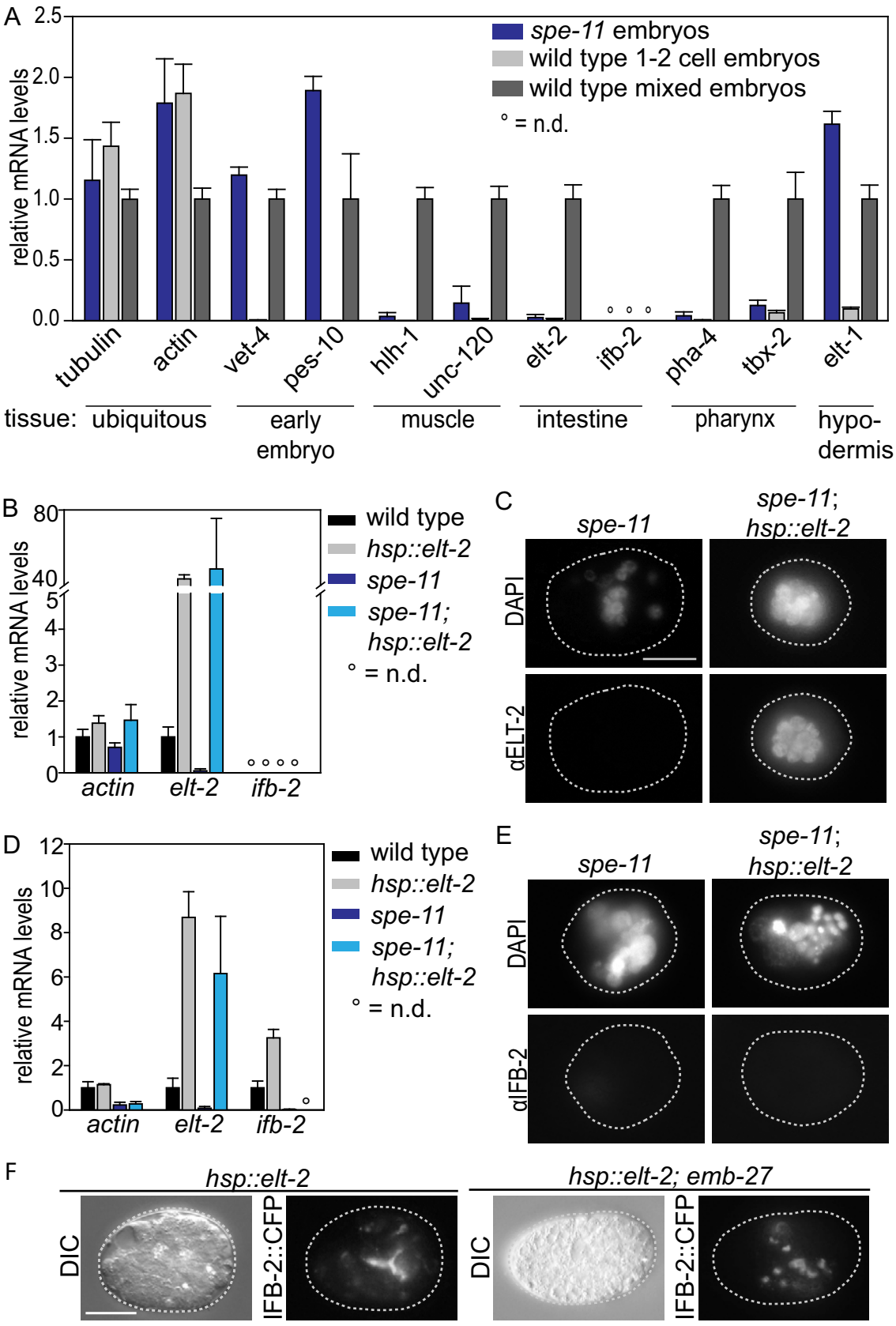


Figure 18: *spe-11* embryos do not differentiate
SPE-11 is the only known sperm delivered protein which functions in the early embryo. *spe-11* embryos fail to build a functional eggshell but otherwise inherit all paternal factors. (A) RNA expression analysis detected the early embryonic

genes *vet-4* and *pes-10* in *spe-11* embryos but from the analyzed tissue specific genes only *elt-1* was expressed which might also be due to polyspermy. (B) 1 h after heat shock induced overexpression of ELT-2, *elt-2* mRNA was highly expressed in *spe-11; hsp::elt-2* but not in control *spe-11* embryos. (C) ELT-2 protein was further expressed in *spe-11; hsp::elt-2* mutants. (D) After 6 h recovery, *elt-2* mRNA was still upregulated in *spe-11; hsp::elt-2* embryos but the terminal gut differentiation marker *ifb-2* was not expressed on mRNA level. (E) The IFB-2 protein was not detectable in *spe-11; hsp::elt-2* and *spe-11* control embryos. (F) In sperm, SPE-11 localizes to chromatin and might lead to chromatin modifications in the paternal nucleus. *emb-27* embryos derived from anucleate sperm, however, were able to respond to a induced cell fate switch. Scale bars represent 20 µm.

through cytokinesis. Since both, *zen-4* and *mei-1* mutant embryos differentiate, the latter two processes can already be ruled out to cause the inability to differentiate. Therefore, we analyzed eggshell mutants for their ability to differentiate.

Eggshell formation depends on a variety of proteins which spatially and temporally coordinate the building of the three eggshell layers and the extra embryonic matrix (Johnston and Dennis, 2011). We chose to analyze *egg-3* mutants which are unable to form any protective eggshell layers leading to very fragile embryos, *gna-2* mutants which fail to build the chitin and subjacent layers and *sqv-4* mutants which are unable to generate the extra embryonic matrix and exhibit mild eggshell defects (Bembenek et al., 2007, Maruyama et al., 2007, Hwang and Horvitz, 2002, Johnston et al., 2006). *vet-4prom::gfp* reporter embryos derived from either *egg-3, gna-2* or *sqv-4* RNAi treated worms expressed GFP at normal levels (Figure 19A). We next performed RNAi on *hsp::elt-2; ifb-2::cfp* embryos, heat shocked the embryos and checked for IFB-2::CFP expression after over night recovery. Most of the analyzed embryos did not express IFB-2::CFP, although for each RNAi condition very weakly expressing embryos were detected - *egg-3* (RNAi): 2 of 11; *gna-2* (RNAi): 20 of 41; *sqv-4* (RNAi): 3 of 23 (Figure 19B). For the majority of these IFB-2::CFP expressing embryos the weak CFP expression coincided with fluorescence through the red channel (*egg-3* (RNAi): 2 of 2; *gna-2* (RNAi): 16 of 20; *sqv-4* (RNAi): 3 of 3). This fact most likely indicates dying embryos as it was never observed in otherwise wild type *hsp::elt-2; ifb-2::cfp* embryos and arises from the problem that these mutants could not be

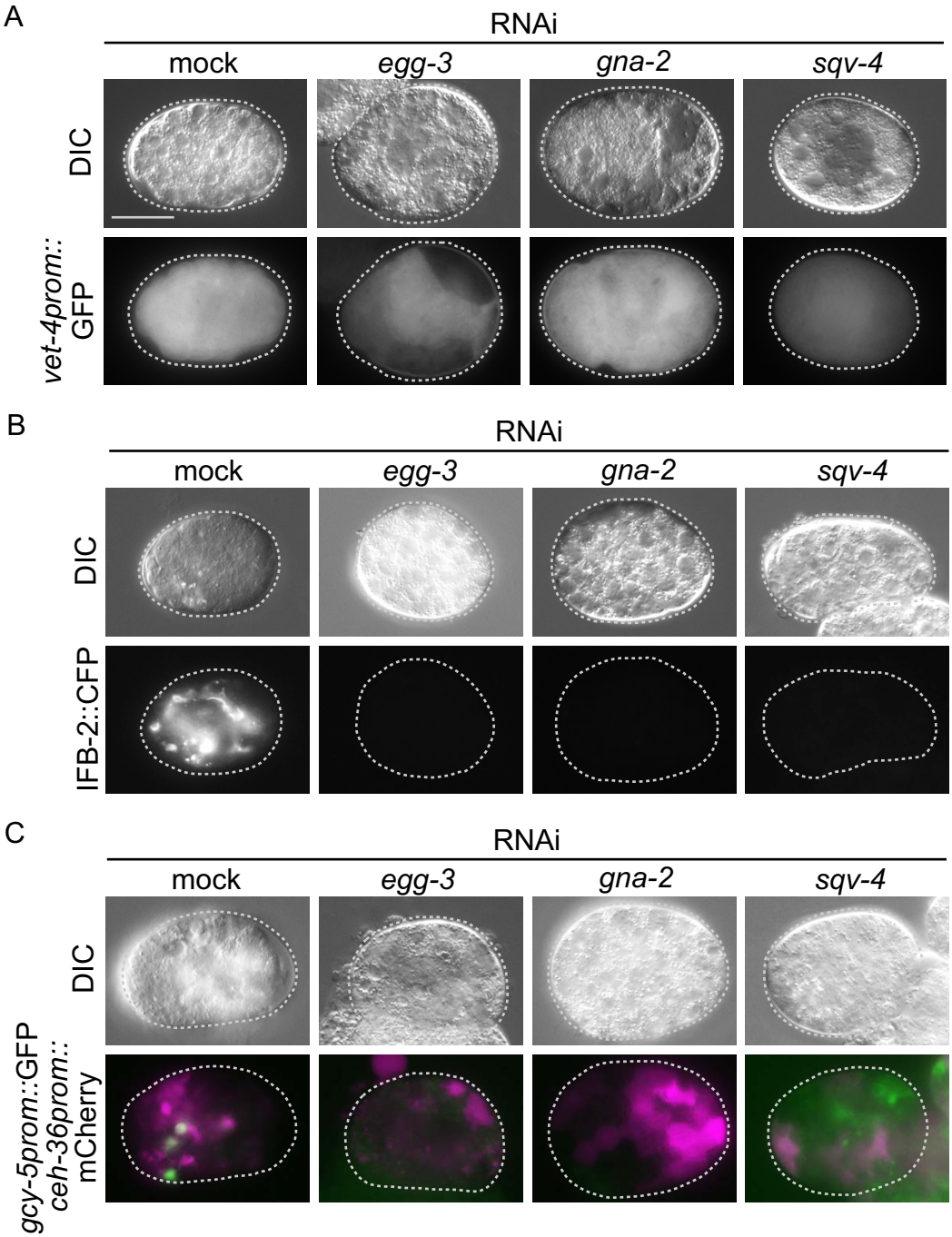


Figure 19: A functional eggshell is partially needed for the ability to differentiate

Eggshell formation is triggered by sperm entry. RNAi against proteins important for different eggshell layers exhibit different severities of eggshell defects ranging from no eggshell layers in *egg-3*, defective chitin and subjacent layers in *gna-2* and no extra embryonic matrix in *sqv-4* mutants. (A) All these mutants expressed the *vet-4prom*::GFP. (B) However, none of these mutants expressed the gut terminal differentiation marker IFB-2::CFP after overexpression of the gut determinant ELT-2. (C) When the neuronal cell fate switch was induced, all eggshell mutants expressed at least one of the two integrated neuronal markers. Scale bar represents 20 µm.

synchronized for a certain age due to their 1-cell stage arrest. Therefore, among the heat shocked embryos will be older ones which will eventually stop developing and die. Given the overall strongly reduced CFP expression, we conclude that eggshell formation is needed for the induced switch towards the gut cell fate.

We next analyzed whether this is specific for the gut cell fate and induced neuronal differentiation. To our surprise, failure in eggshell formation did not prevent neuronal differentiation as we detected embryos which strongly expressed at least one neuronal marker in all RNAi conditions. The fraction of neuronal marker expressing embryos seemed to be related to the severity of the eggshell mutation. In severely affected *egg-3* (RNAi) embryos, 2 of 17 embryos express neuronal markers. Of the less severely affected eggshell mutations, 13 of 19 *gna-2* (RNAi) embryos and 15 of 15 *sqv-4* (RNAi) embryos strongly expressed neuronal markers after heat shock (Figure 19C).

These findings suggest that eggshell formation is partially needed for the ability to differentiate into certain lineages.

4. Discussion

4.1. *vet-4* reporter strain and the role of chromatin modifiers in EGA

In order to visualize embryonic gene activation (EGA) in *C. elegans*, we created a reporter strain and chose the gene *vet-4* as a marker. *vet-4* transcript abundance in the early embryo has been analyzed in several studies although VET-4 function remains speculative (Seydoux et al., 1996, Baugh et al., 2003). Further, amongst all analyzed early embryonic genes, *vet-4* RNA levels were highest in developing teratomas and a *vet-4* reporter strain therefore helpful for several projects (Figure 8A and Biedermann et al., 2009). We fused a 3 kb *vet-4* promoter fragment to a NLS containing *gfp* ORF followed by the *vet-4* 3' UTR.

vet-4prom::GFP expression nicely mimicked the onset of EGA in *C. elegans* and is therefore a potent tool to visualize early embryonic gene expression. Early during our analyses we discovered discrepancies between expected and analyzed results. First, despite an included NLS, GFP was uniformly expressed in embryonic cells. The NLS coding sequence is known to lead to nuclear localization of several analyzed proteins in *C. elegans* and although it is most often used as a C-terminal tag, also functions at the N-terminus. The problem therefore seems to lie in the GFP protein itself. And indeed, GFP has a size of approximately 27 kDa but passive diffusion through the nuclear pore complex is possible for proteins up to 60-90 kDa (Wang and Brattain, 2007). We therefore hypothesize that the NLS::GFP is actively translocated into the nucleus but due to the high concentration difference passively diffuses back to the cytoplasm. Although cytoplasmic GFP contribution was unexpected, it is not detrimental for further analyses.

Second, the *vet-4* reporter showed differences between expression levels of endogenous *vet-4* and transgenic *vet-4prom::gfp* mRNAs for *mep-1* (RNAi) embryos. In a pilot RNAi screen performed together with Cristina Tocchini, 22 of 61 analyzed genes showed a GFP expression pattern similar to *mep-1* (RNAi) embryos. This unexpected high number of potential EGA regulators was puzzling and initial experiments suggested a similar problem as for *mep-1*

(RNAi). There are several possible reasons which might cause this discrepancy. On the one hand, during the cloning process, two point mutations were introduced into the promoter region. Both mutations lead to an A-to-G substitution within a stretch of several adenosines. Although both point mutations are relatively far upstream the coding sequence, they might influence the accessibility of transcriptional activators or repressors. Further, it has been shown that promoter sequence motifs may have an influence on mRNA stability (Bregman et al., 2011, Trcek et al., 2011). Although the described motifs are not found in the mutated *vet-4* promoter region, other, yet unidentified, motifs might have been altered. More generally, a minimum promoter sequence has not been analyzed and it might be possible that the 3 kb promoter does not contain all necessary enhancer motifs as they might lie further upstream or within the *vet-4* ORF. On the other hand, the *vet-4* reporter strain was created by bombardment which leads to multicopy random integration or extrachromosomal arrays of the transgenes. In total, five *vet-4prom*::GFP expressing strains could be identified from which only one behaved like an integrated strain. All five strains were initially analyzed and no phenotypical difference was observed between the non-integrated and the integrated strains so that final experiments were performed on the integrated strain only. It is, however, unclear at which genomic position the *vet-4* reporter plasmid integrated and it might be possible that the integration event itself destroyed another gene or regulatory element. In any case, neither the point mutations nor the disrupted genomic region have an effect on wild type embryos as *vet-4prom::gfp* expression mimics endogenous *vet-4* levels. However, knockdown of certain genes might generally affect nuclear proteome composition which now demasks differences in endogenous and transgenic *vet-4* expression.

The disadvantage of random integration can now be overcome by single-copy integration into a defined locus using the MosSCI technique (Frokjaer-Jensen et al., 2008). Together with Cristina Tocchini, we fixed the nuclear localization problem by fusing the NLS to a GFP dimer and created a single copy integrated *vet-4* reporter strain. Interestingly, *mep-1* (RNAi) on the single copy integrated line still led to differences in the expression pattern compared to

mock (RNAi) treated embryos. The differences were, however, less strong, indicating that a combination of the above mentioned factors led to the discrepancies.
Taken together, the *vet-4* reporter strain is a suitable tool for analyzing the onset of EGA, but differences in later stage embryos have to be carefully analyzed if RNAi or mutations are involved. The single copy integrated *vet-4* reporter strain is now used to identify EGA regulators by a classical mutagenesis screen.

EGA regulators in other model systems include chromatin modifiers such as the nucleosomal remodeling SWI-SNF complex member Brg1 in mouse (Bultman et al., 2006). In *C. elegans*, chromatin modifiers are important for a variety of developmental processes including maintenance of the germ line versus soma differentiation. In this context, the PRC2 complex members MES-2, MES-3 and MES-6 together with the H3K36 methyltransferase MES-4 counteract the MEC complex members MEP-1, HDA-1 and LET-418 (Unhavaithaya et al., 2002). In order to identify chromatin modifiers which are essential for the onset of EGA, we performed an initial screen composed of these genes and analyzed *vet-4prom*::GFP expression. Knockdown of these genes, however, did not lead to changes in GFP or endogenous *vet-4* expression. Therefore, we conclude that neither the MEC complex nor the PRC2 complex or MES-4 are involved in regulating the onset of EGA in *C. elegans*. These findings, however, do not exclude the involvement of additional chromatin modifiers. The multicopy integrated *vet-4* reporter strain is not suitable for an expanded RNAi screen, but chromatin modifiers might be detected as EGA regulators in the above mentioned ongoing mutagenesis screen.
Generally it might be questionable whether chromatin modifiers will have enough time for their action during the initial two cell cycles. The four cell stage embryo develops within 60 minutes after fertilization. If chromatin modifiers play an essential role during EGA onset, they should not be active in the oocyte before fertilization and thus need to be either translated or activated in the zygote. This process potentially is time consuming as well, so

that the time of action will be less than 60 minutes. This is in strong contrast to mouse development in which chromatin modifiers have been identified as EGA regulators. EGA starts in the mouse already at the pronuclear stage but given that the first cell division needs approximately 24 h, even at the pronucleus stage quite some time passed in which Brg1 could act. Therefore, it might be more likely that epigenetic modifications in *C. elegans* are important during later stages of embryogenesis but not necessarily for the onset of EGA.

4.2. Teratoma formation involves precocious embryonic-like differentiation

In a previous study, teratoma formation has been shown to require a precocious switch from meiosis to mitosis (Biedermann et al., 2009). Normally, germ cells re-enter the mitotic cell cycle after fertilization which is a fundamental process during early embryogenesis. And indeed, once germ cells precociously re-entered mitosis, they start expressing early embryonic genes (Biedermann et al., 2009). We therefore hypothesized that teratoma formation equals pre-mature embryonic development and tested whether hallmarks of early embryogenesis are occurring in the developing teratoma. These hallmarks include a phase of transcriptional inactivation, switching from germline to somatic mode, expressing early embryonic genes followed by lineage specific genes and reaching a cellular plasticity to allow tissue specification.

Previously, it has been shown that teratomatous cells lose germ line specific P-granules and the expression of somatic muscle and neuronal markers indicate a successful switch from a germline to a somatic environment (Biedermann et al., 2009, Ciosk et al., 2006).

Wild type embryogenesis involves transcriptional re-activation of transcriptional silent gametes and early embryonic nuclei. In the teratomatous germ line, re-entry into mitosis and subsequent proliferation occur randomly and uncontrollably. It is therefore impossible to analyze teratoma formation in a spatially or timely restricted fashion. However, teratoma formation can be completely blocked by cyclin E/*cye-1* (RNAi) which prevents germ cells from re-entering mitosis. By comparing young teratomatous gonads in which teratoma formation just started with gonads in which teratoma formation is blocked, we could identify 35 % of transcriptional silent cells in young teratomatous gonads. The frequency of transcriptional silent cells decreased to 3 % in gonads in which teratoma formation is blocked. These findings suggest that a transcriptional silent phase precedes teratoma formation similar to wild type embryogenesis. Proliferating teratomatous cells were

transcriptionally active. The block in teratoma formation does not alter the number of analyzable cells in the central part of the gonad. Further, it is unlikely that the high number of transcriptionally silent nuclei in the teratoma represent dying cells. The transcriptionally silent cells are phenotypically not distinguishable from surrounding transcriptionally active cells whereas cells undergoing apoptosis within wild type gonads exhibit a dramatically different cell morphology.

In wild type embryos, EGA allows the expression of early embryonic genes followed by lineage specific genes as soon as cells differentiate. The majority of the early embryonic genes was also expressed in the two analyzed teratoma strains (*vet-4, vet-6, pes-10*). However, not all of the tissue specific genes could be detected and a clear preference towards muscle and pharyngeal tissues arises. For muscle differentiation, the preference seems to origin solely from the presence of certain tissue specific transcription factors, e.g. PAL-1. *pal-1* mRNA is expressed in the wild type germ line but translationally repressed by GLD-1 and MEX-3. In the teratoma, GLD-1 is no longer present and *pal-1* mRNA is therefore likely to be translated and ready to initiate muscle differentiation.

By inducing a cell fate switch towards the otherwise rarely occurring gut cell fate, it could be shown that teratomatous cells are plastic and able to differentiate into the desired fate. In wild type development, only early embryonic cells are able to adopt an induced cell fate switch which indicates that teratomatous cells are similar to early embryonic cells.

Taken together, comparing wild type embryogenesis and teratoma formation revealed that the teratomatous development follows embryogenesis. As these experiments have been performed in two different teratomatous backgrounds, precocious embryogenesis seems to generally cause teratoma formation in *C. elegans*.

Pre-mature embryogenesis has also been described to cause teratoma formation in females of the Mos -/- mouse strain. Teratomas frequently develop in the ovaries of these mice and early pre- and post-implantation embryos could be detected in ovaries (Hirao and Eppig, 1997). Mos -/-

teratomas are thought to originate from pre-maturely activated oocytes within the ovary. In contrast, in *C. elegans*, re-entry into mitosis occurs before oocytes are formed and teratoma formation cannot occur simply as a result of oocyte activation. Germ cells which re-enter mitosis are smaller than mature oocytes. And although the proteomic and transcriptomic components might be similar to oocytes, these germ cells differ at least in their yolk amount and the presence of centrosomes. Yolk intake depends on the yolk receptor RME-2 which is usually expressed in developing oocytes in the proximal gonad. Although *rme-2* is a target of GLD-1, RME-2 protein is not detected in *gld-1* dependent teratomas (Ciosk et al., 2006). On the other hand, as the teratoma does not develop within a closed environment like the embryo in its eggshell, it might not depend on intrinsic nutrients. Further, mature oocytes lost their functional centrosomes and depend on sperm derived centrioles to form centrosomes in the embryo. As centrosome reduction occurs at a later stage during oogenesis, they are still present in germ cells which re-enter mitosis and eventually differentiate. However, whether centrosomes play a crucial role in allowing differentiation remains speculative.

4.3. Unfertilized oocytes show early embryonic features but a yet unknown sperm associated event licenses lineage specific transcription

In the sexually reproducing nematode *C. elegans*, no evidence of parthenogenetic development exists. However, previous studies suggested that ovulated but unfertilized oocytes exhibit embryonic rather than germline features (McNally and McNally, 2005, Ward and Carrel, 1979). However, no detailed analysis of embryonic development within unfertilized oocytes has been described.

We show that unfertilized oocytes lost germ line characteristics and acquired somatic features. The transcriptional inhibitor OMA-1::GFP was degraded in unfertilized oocytes and transcription was reinitiated. Interestingly, active transcription could only be detected in unfertilized oocytes which underwent endoreplication. Whether onset of endoreplication is necessary for transcriptional reactivation, e.g. through a change in the DNA - cytoplasm ratio which allows titration of transcriptional repressors, or whether transcriptional reactivation solely depends on OMA-1/2 degradation remains elusive. We further detected the early embryonic transcript *vet-4* in unfertilized oocytes and both, *vet-4* and *pes-10* reporter strains, expressed GFP. However, no tissue specific gene could be detected which indicates that unfertilized oocytes do not differentiate. Further, unfertilized oocytes were unable to respond to induced cell fate switches and thus generally lack the ability to differentiate, as has been shown for gut and neuronal induced cell fate switches. These findings suggest that first steps of embryogenesis are taking place in unfertilized oocytes. However, probably due to the lack of centrosomes and the inability for acentrosomal spindle formation, unfertilized oocytes do not divide. Therefore unfertilized oocytes cannot be referred to as “parthenotes”.

We further investigated the reasons for the inability to differentiate. Unfertilized oocytes progress through an abnormal meiosis, have failures in cytokinesis, lack sperm derived components and the fertilization induced eggshell formation. We analyzed mutants defective for meiosis and cytokinesis and found that they were able to differentiate and induce a cell fate switch. Therefore, impaired meiosis and missing cytokinesis did not cause the failure to differentiate in unfertilized oocytes but sperm associated factors seem to license the development into a differentiation competent stage during embryogenesis.

To distinguish between sperm inherited components and sperm dependent eggshell formation, we analyzed *spe-11* embryos. SPE-11 is a sperm derived protein which is essential during early embryogenesis when it is needed for creating a functional polyspermy barrier and subsequent eggshell layers. *spe-11* mutant embryos therefore phenotypically resemble unfertilized oocytes with the main difference that they contain all sperm derived components and undergo nuclear divisions. Surprisingly, RNA expression analysis detected only one tissue specific gene in *spe-11* mutants - the hypodermal factor *elt-1*. Unfortunately, *elt-1* has an additional function during spermatogenesis and it is unclear whether the detected *elt-1* mRNA is embryonically expressed or originates from polyspermy. Yet, *spe-11* embryos did not respond to an induced cell fate switch towards the gut lineage, although the *elt-2* mRNA could be detected longer than in unfertilized oocytes.
Taken together, although *spe-11* mutants derive from fertilized oocytes and inherit functional sperm components, they are unable to develop into a differentiation competent stage. Sperm specific inherited factors are therefore largely dispensable for the embryonic differentiation ability.

A striking similarity between *fem-1* unfertilized oocytes and *spe-11* embryos is the missing eggshell. We analyzed three genes which play fundamental roles in eggshell formation and checked whether mutant embryos are able to differentiate. Knockdown of the genes leads to different degrees of eggshell defects ranging from complete absence (*egg-3*) to the inability to fill the extra

embryonic matrix with fluid (*sqv-4*). After cell fate switch induction, eggshell mutant embryos had severe problems in generating IFB-2::CFP as a marker for gut differentiation. In fact, only a minor fraction of the embryos showed any CFP expression and in most of these cases it is questionable whether the expression comes from arrested or dying embryos. When the neuronal cell fate was induced, most embryos were expressing the neuronal markers. However, in the case of *egg-3* (RNAi) only 2 of 17 embryos expressed the markers, indicating a strongly reduced ability to respond to the induced cell fate switch. A functional eggshell therefore might facilitate the development into a differentiation competent state.

These analyses have been performed with RNAi and although RNAi efficiency has not been quantified, we clearly detected the expected phenotypes for all analyzed knockdowns. Whether some embryos expressed the markers due to insufficient knockdown of the genes is unlikely but cannot be ruled out. In a controlled heat shock experiment, embryos are synchronized by dissecting them at the one- to four-cell stages and let them develop until the recommended time before they are exposed to heat shock. This ensures that all the embryos are in the same developmental stage and similarly react to the heat shock. For all analyzed eggshell and cytokinesis defective mutants, synchronization was not possible as embryos arrest at the 1-cell state and look similar under the microscope. Minimal synchronization of these embryos and unfertilized oocytes was achieved by dissecting *in utero* embryos out of very young adult worms. However, variations in the ability to react to the induced cell fate switches might be partially due to age differences of these embryos.

In summary, both, unfertilized oocytes and fertilized *spe-11*, embryos are unable to respond to an induced cell fate switch. Assuming that *spe-11* sperm contains functional components at wild type levels, these components are not needed for the embryonic ability to differentiate. However, a functional eggshell which separates and protects the developing embryo from its surrounding environment seems to contribute to the embryonic differentiation

ability. As one of the two analyzed induced cell fate switch completely relied on a proper eggshell and the other cell fate switch partially depended on it, we conclude that a functional eggshell contributes to the embryonic ability to differentiate.

Generally for unfertilized oocytes, although a transcription factor is functionally expressed after heat shock it does not trigger the expression of its target genes which might be indicative for its inability to access and bind to promoter regions. The retention from its target DNA can be achieved by repressors which block the binding sites or epigenetic modifications which are established during gametogenesis and require specific remodeling. Indirect evidence for the latter example arises from a study in which a cell fate switch was induced in the hermaphrodite germ line in several knockdown situations. If the gene *lin-53* was absent, germ cells were able to differentiate into neurons. Interestingly, no other somatic fate could be induced which indicates that germ cells are poised for neuronal development (Tursun et al., 2010). Generally, several gene knockout studies in *C. elegans* and vertebrates indicate that many if not all undifferentiated cells have a “neuronal ground state” which is actively repressed and the removal of the repressor is sufficient to induce neuronal differentiation (reviewed in Hobert, 2010). If germ cells posses this neuronal ground state, factors or pathways will exist which release repressors in embryos. Neuronal differentiation in the wild type embryo occurs only late during embryogenesis and depends on the presence of neuron specific transcription factors. The transcription factor CHE-1 is not present in gonads and the early embryo, but is ectopically introduced by our technique. Thus, the fact that the neuronal fate is not induced in ovulated oocytes but in eggshell mutants might indicate that the release of any neuronal repressor solely depends on post-fertilization events whereas the differentiation into e.g. the gut fate involves major nuclear remodeling and therefore depends on additional factors or processes like the eggshell formation. Interestingly, the CBP/p300 homolog CBP-1 with its histone acetyltransferase activity is known to promote all non-neuronal pathways of somatic differentiation in the embryo

(Shi and Mello, 1998). CBP-1 is expressed early during embryogenesis and might therefore serve as a major nuclear remodeling factor.

Ovulated but unfertilized oocytes, however, do not show any sign of differentiation. These oocytes are not activated as oocyte activation in *C. elegans* requires fertilization. The calcium wave during oocyte activation leads to activation of a variety of processes and it is speculative to assume that one of these processes might release the general block of neuronal differentiation whereas an additional process initiates nuclear remodeling and allows gut differentiation. In this context it would be interesting to know whether *spe-11* mutants, which fail to induce the gut cell fate, would be able to induce the neuronal cell fate. So far, however, we struggle to cross these strains and RNAi against *spe-11* is impossible.

Therefore, we propose the preliminary model (Figure 20) in which activation of early embryonic genes solely relies on oocyte maturation, whereas the expression of tissue specific genes requires fertilization which activates an unknown factor X in the case of ectopic neuronal development and fertilization in combination with eggshell formation and an unknown factor Y in the case of ectopic gut differentiation. However, several questions and analyses remain before a satisfying model can be build and the mechanism which leads to the acquisition of embryonic differentiation is dissected in detail.

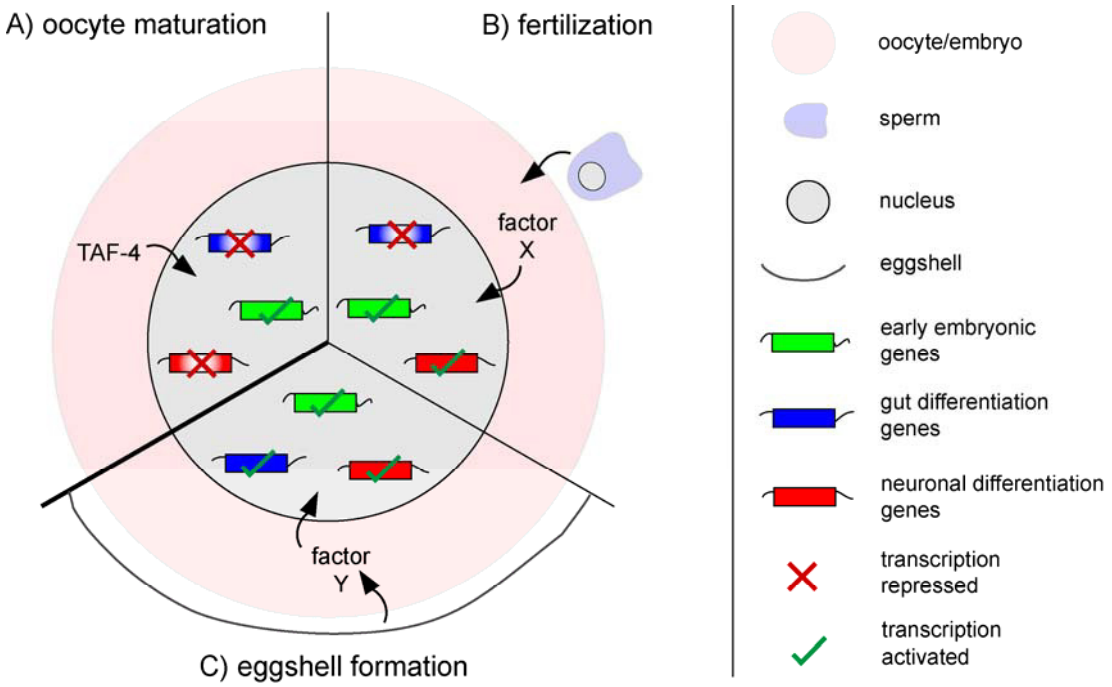


Figure 20: Preliminary model for acquiring the ability to differentiate
(A) Expression of early embryonic genes depends on oocyte maturation and the nuclear translocation of TAF-4. (B) Fertilization allows the ectopic expression of neuronal differentiation genes, potentially through an additional factor or process “X”. (C) Fertilization induced eggshell formation allows the ectopic expression of gut differentiation genes, potentially through activating a factor or process “Y”.

4.4. General discussion

In *C. elegans*, as in most other species, maternally derived factors are sufficient to initiate first steps of embryogenesis. In ovulated but unfertilized oocytes, embryonic-like features include both MET events - mRNA degradation and onset of EGA. In contrast to most other species, however, *C. elegans* embryos depend on sperm or fertilization derived factors early during their development as unfertilized oocytes do not develop past a stage similar to the four-cell stage embryo. *C. elegans* embryos depend on sperm centrioles as neither acentrosomal spindle nor *de novo* centriole formation has been reported. Unfertilized oocytes are therefore unable to divide and remain in an endoreplicating one cell stage. Centrosomes are known to accumulate proteins which are necessary for proper mitosis but whether centrosomes or accumulated proteins are important for embryonic differentiation remains unknown. From our studies in *spe-11* mutants in which centrosomes are present, it seems unlikely that centrosomes play a major role in embryonic differentiation. However, an initial knockdown of two centrosome associated proteins in otherwise wild type embryos resulted in a reduced ability to differentiate. It might therefore be interesting to analyze the embryonic ability to differentiate in centrosome mutants. Another link towards centrosomes comes from comparing teratomatous embryogenesis with unfertilized oocytes. Teratomatous cells are able to differentiate and in contrast to unfertilized oocytes, still contain centrosomes. On the other hand, teratoma formation is initiated at a stage in which most chromatin and cytoplasmic modifications which might be present in mature oocytes have not yet been established. Teratomatous cells might therefore have an advantage for initiating embryogenesis. Further, although we show that a non functional eggshell negatively influences the embryonic ability to differentiate, teratomatous cells do not build an eggshell. The eggshell in wild type embryos might simply create a barrier between the environment and the developing embryo but especially the fluid filled extra embryonic matrix might serve as a specialized signaling compartment as has been shown for *D. melanogaster* dorsal-ventral

patterning (Stein and Nusslein-Volhard, 1992). During teratoma formation, a signaling pathway is either not needed or exists already e.g. through the somatic gonad.
Eggshell formation and its influence in embryogenesis seems to be restricted to invertebrates like nematodes and flies. However, also early mammalian embryos develop initially within a specialized microenvironment enclosed by the zona pellucida and an underlying cortical granule envelope (Dandekar and Talbot, 1992). Thus, creating an eggshell-like structure seems to be a general and essential feature in early embryogenesis across species.

In *C. elegans*, MET onset can be uncoupled from oocyte activation and fertilization as it solely depends on oocyte maturation. Oocyte maturation is initiated by sperm derived MSP and thus generally allows oocyte maturation only in the presence of sperm. Parthenogenetically reproducing nematodes do not produce MSP, yet oocyte maturation occurs. This suggests that the MSP dependence can be easily overcome and backup mechanisms must exist to ensure the sole survival of biparental embryos in *C. elegans*. Sperm specific centrosome inheritance might function as a backup mechanism although parthenogenetically reproducing nematodes apparently are able for either acentrosomal spindle or *de novo* centrosome formation. Thus, another backup mechanism might involve eggshell formation. And indeed, in parthenogenetically reproducing nematodes, eggshell formation takes place only after oocytes are laid to their environment. In the case of *Plectus* sp., embryonic development does not occur *in uteru* if egg laying is blocked. It is assumed that a maternal signal inhibits embryonic development and only after the release from the uterus, the maternal signal vanishes and embryogenesis is initiated. In *C. elegans*, eggshell formation after fertilization might therefore rapidly block maternal signals from interfering with embryogenesis.
In *C. elegans*, very early embryonic genes are expressed in unfertilized oocytes. The functions of these early embryonic genes are unknown but are predicted to mediate protein-protein interactions and ubiquitin mediated protein degradation. Apparently, in unfertilized oocytes these genes alone are not sufficient for pursuing embryogenesis but this might be due to missing

substrates or interaction partners which expression or activation depend on other mechanisms, e.g. oocyte activation. Parthenogenetically reproducing nematodes must be able to activate their oocytes independently of fertilization and might therefore be able to overcome this block.

In summary, fertilization uncoupled onset of MET might support the formation of parthenogenetic reproduction. Generally, it is believed that parthenogenesis originated from sexual reproduction and not vice versa. Thus, it is very likely that fundamental processes during embryonic development are similar in related species. In flies, for example, oocyte activation is independent of fertilization which supported the creation of parthenogenetic species. In nematodes, early embryonic features like maternal RNA degradation and EGA onset occur independently of fertilization which might have facilitated the evolution of parthenogenetically reproducing nematode species. In sexually reproducing species, like *C. elegans*, however, additional restrictions, such as sperm induced eggshell formation, prevent parthenogenetic development.

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6. Appendix

6.1. Abbreviations

A-P axis	anterior-posterior axis
CPEB	cytoplasmic polyadenylation-element-binding protein
DTC	distal tip cell
EGA	embryonic gene activation
GO	gene ontology
H3K27m3	tri-methylation of histone 3 lysine residue 27
H3K36m	methylation of histone 3 lysine residue 36
IRC	imprinting control region
IVF	*in vitro* fertilization
L1 (2, 3, 4)	larval stage 1 (2, 3, 4)
M-Z-	maternal homozygous mutant, zygotic homozygous mutant
MEC	MEP-1 containing (complex)
MET	maternal to embryo transition
miRNA	micro RNA
MSP	major sperm protein
MTOC	microtubule organization center
NLS	nuclear localization sequence
NuRD	nucleosome remodeling and histone deacetylase (complex)
ORF	open reading frame
PIC	polymerase II pre-initiation complex
piRNA	piwi-interacting RNA
Pol II	polymerase II
PRC2	polycomb repressive complex 2
P-Ser2 (5)	phosphorylated serine residue 2 (5) of Pol II CTD subunit
STAR	signal transduction and activation of RNA
SWI-SNF	switch-sucrose non-fermentable (complex)

6.2. List of figures

6.3. List of tables

6.4. Acknowledgements

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