Regulation of totipotency in the *Caenorhabditis elegans* germ line

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Basel, den 02.03.2010

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Dekan
(Prof. E. Parlow)

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Table of content

1) INTRODUCTION

a. Germ cell development.

i. Germ cells form the only totipotent cell, the zygote, but germ cells also have an underlying totipotency.

Sexual reproduction of an individual animal and hence its ability to pass its genetic information to the next generation typically lies in the formation of specific cells, the germ cells. The aim of germ cell specification is the formation of highly differentiated cells, oocyte and sperm. The fusion of an oocyte and a sperm leads to the formation of the zygote which has the potential to differentiate into each cell type and can form a whole organism. The potential to differentiate into any cell type is defined as totipotency (Seydoux and Braun 2006).

Germ cells have an underlying totipotency. This high developmental potential of germ cells can manifest in an unusual germline tumor, called teratoma. This tumor contains various types of terminally differentiated somatic cells, such as muscle, neurons, hair, bones (Ulbright 2005). Furthermore the underlying totipotency of germ cells becomes obvious by the possibility to derive pluripotent cell lines from various types of germ cells, such as primordial germ cells (PGCs), or spermatogonial stem cells. These pluripotent cells have the ability to differentiate into various somatic cell types derived from the three germ layers *in vitro,* and *in vivo*. And importantly in chimeric animals, they are able to contribute to the germ line, and hence to form an organism (Kerr et al. 2006).

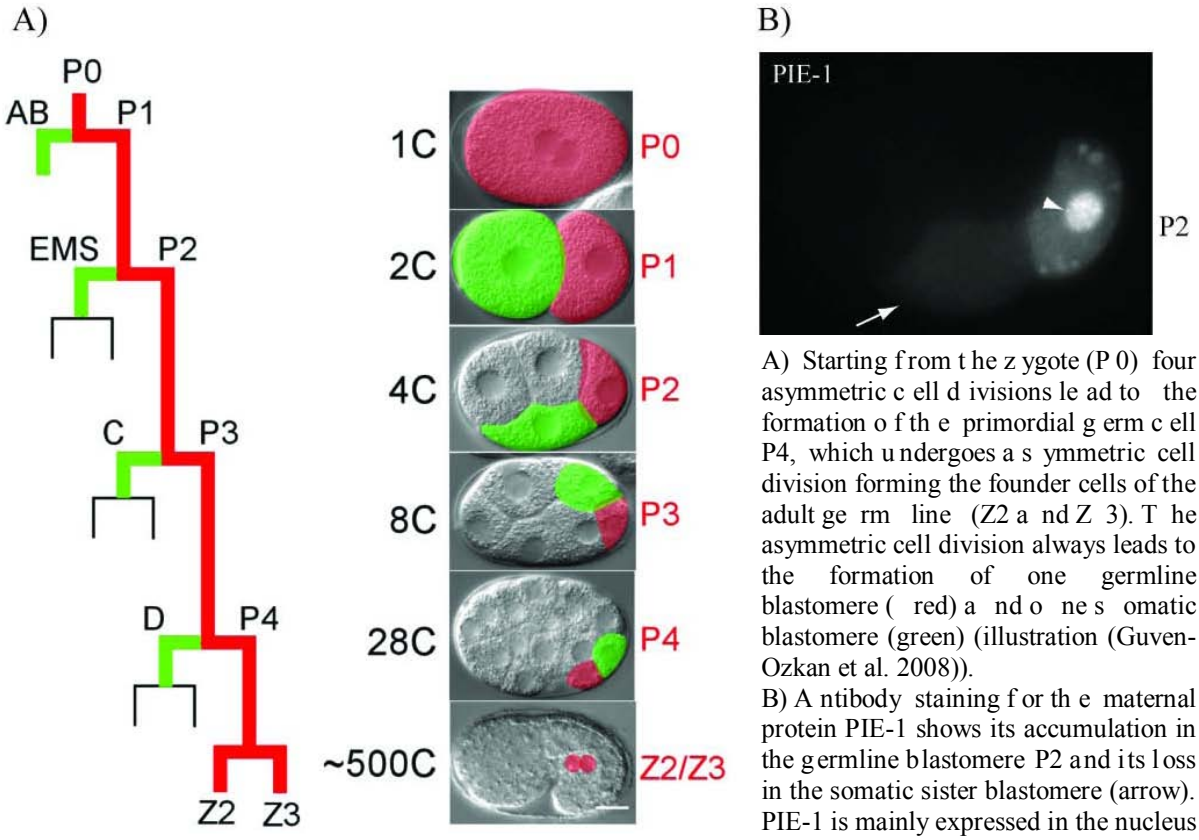
This leads to the interesting question of what the mechanisms are that allow germ cells to differentiate into highly specific cells, while maintaining/forming an underlying totipotency. What are the mechanisms that induce germ cell specification, are required to establish and to maintain germ cell identity, and promote germ cell differentiation, while keeping these cells competent for somatic differentiation?

ii. Primordial germ cell specification.

Depending on the organism the specification of the embryonic precursors of the female and male gametes, the primordial germ cells, can occur through two different modes, “epigenesis” and “preformation”. In “epigenesis” germ cells are induced through signals from surrounding cells, while in “preformation” maternally provided germ cell determinants are localized to one specific cell in which they promote germ cell formation (Extavour and Akam 2003). Germ cell development can roughly be divided into three phase: Primordial germ cell (PGC) specification, gonad colonization, and gametogenesis.

In *C.elegans* the germ line is constantly maintained throughout the life cycle of the worm. Through preformation the germ line is already defined in the totipotent zygote (P0), and a first asymmetric cell division leads to the formation of one somatic blastomere (AB cell), and one germline blastomere (P1 cell). The germline blastomere undergoes three more asymmetric cell divisions, leading to the formation of always one somatic, and one germline blastomere (P2 and P3). The last division creates the primordial germ cell P4, which undergoes a symmetric cell division forming the founder cells of the adult germ line, Z2 and Z3. These cells stay in the gonadal primordium in a quiescent state till after hatching (Fig.1A) (Sulston et al. 1983).

Fig. 1) Maternally provided factors, which are segregated through assymetric cell divisions, define the germ line during embryogenesis in C.elegans.

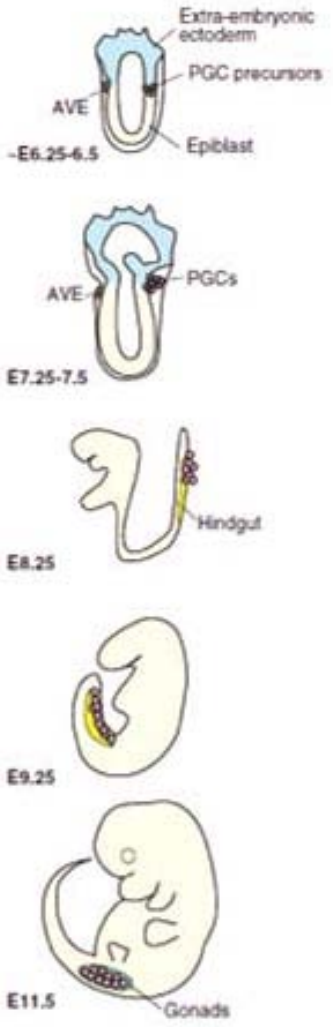


A) Starting from the zygote (P0) four asymmetric cell divisions lead to the formation of the primordial germ cell P4, which undergoes a symmetric cell division forming the founder cells of the adult germ line (Z2 and Z3). The asymmetric cell division always leads to the formation of one germline blastomere (red) and one somatic blastomere (green) (illustration (Guven-Ozkan et al. 2008)).
B) Antibody staining for the maternal protein PIE-1 shows its accumulation in the germline blastomere P2 and its loss in the somatic sister blastomere (arrow). PIE-1 is mainly expressed in the nucleus and in P-granules (e.g. arrowhead), which are attached to the nuclear envelope (image (Strome 2005)).

In the mouse at E6-6.5 (embryonic days) PGC specification is induced in pluripotent cells of the proximal epiblast through signals from surrounding cells of the endoderm and the extraembryonic ectoderm (Lawson and Hage 1994; Lawson et al. 1999; Ying and Zhao

2001). Initially PGC formation is induced in approximately 6 cells, which undergo specification and proliferation forming a small cluster of approximately 40 PGCs at the base of the developing allantois at E7.5 (Ohinata et al. 2005; Payer et al. 2006). PGCs continue to proliferate and start to migrate through the developing hindgut in order to populate the primordia gonad at E10.5-11.5 (Wylie 1999), where they form a population of about 26,000 germ cells by E13.5 (Fig.2). Gametogenesis is induced through signals from the fetal gonad governing the choice for a male or female fate of the germ cells. However the completion of meiosis and the formation of functional gametes is influenced by the karyotype of the germ cells. At E13.5 in the developing ovary, germ cells stop to proliferate and arrest in a specialized, prolonged meiotic arrest, called dictyate. In males, germ cells enter a mitotic arrest (G1/G0) at E13.5.

Fig.2 Primordial germ cell specification and migration during mouse embryogensis.



A) During embryonic day (E) 6.25-6.5 signals from the extra-embryonic ectoderm induce primordial germ cell specification in approximately six proximal epiblast cells. These cells migrate to an extra-embryonic location, while undergoing proliferation. By E7.5 they have formed ~40 PGCs, which are located at the root of the allantois and start to migrate back into the embryo in association with the hindgut (yellow) to colonize the primordial gonad by E11.5. AVE: anterior visceral endoderm, (from (Nakamura and Seydoux 2008))

1. Initiation of primordial germ cell formation is different in *C.elegans* and the mouse.

In *C.elegans* P-granules, which are germline specific structures, consisting of proteins and RNAs, are maternally provided by the oocyte and during embryogenesis they are specifically segregated to the germline cells through cell polarization (Fig.1b). P-granules

seem to function as storage granules for proteins and RNAs which are required for germ cell development and embryogenesis; however the exact function of P-granules is not understood (reviewed in (Strome 2005)). Examples of P-granule proteins are the zinc-finger proteins PIE-1, OMA-1, and OMA-2. Both PIE-1, as well as the OMA proteins, are required to establish the *C.elegans* germ line during embryogenesis, as with the loss of these factors the germline blastomeres acquire a somatic fate (Mello et al. 1992; Guven-Ozkan et al. 2008).

In the mouse transplantation experiments (at E6.5) have shown that distal epiblast cells, which would normally give rise to neuroectoderm, can form PGCs, if grafted to the proximal epiblast (Tam and Zhou 1996), suggesting a site-specific influence on PGC specification. Further studies on knock out mutants and genetic chimeras showed that members of the transforming growth factor β (TGF-β) superfamily, bone morphogenetic proteins BMP-2, BMP-4, BMP-8b, induce PGCs formation in the proximal epiblast ((Lawson et al. 1999; Ying and Zhao 2001; Wu and Hill 2009). BMPs are secreted by the surrounding cells of the extraembryonic ectoderm and endoderm and were found to be required for expression of genes marking the onset of germ cell specification, e. g. *fragilis* expression requires BMP4 (Saitou et al. 2002). Single cell analysis showed that these inductive signals change the molecular program of the epiblast cells, which per se are primed for a somatic fate, as indicated by the expression of homeobox genes. PGC specification is preceded and is defined by the sequential expression of several PGC marker genes, such a s tissue non-specific alkaline phosphatase (TNAP), *fragilis*, *blimp-1*, *stella*, and further upregulation of *oct4*, while somatic genes are downregulated (Hoxa1, Hoxb1, Lim1, EVx1) (Saitou et al. 2002).

Taken together both organisms are using different strategies for PGC formation. While in *C.elegans* the localization of germline determinants in the totipotent zygote defines the germline blastomeres and PGCs, in the mouse inductive signals in already lineage committed cells lead to a fate change and to the formation of PGCs.

2. Transcriptional control is a conserved mechanism for primordial germ cell specification.

As in *C.elegans* the loss of OMA proteins or PIE-1 leads to germ line to soma transition, it was of great interest to reveal their molecular function. OMA-1/OMA-2 contain 2 TIS11-like zinc fingers each. They start to be expressed in the cytoplasm of maturing oocytes, and are lost again in the 2 cell embryo (Detwiler et al. 2001). OMA proteins function

in germ line specification, and oocyte maturation (Detwiler et al. 2001; Shimada et al. 2006), and on a molecular level OMA-1 and OMA-2 were found to repress redundantly transcription in the one and two cell stage embryo (= P0 and P1). OMA1/2 apply molecular mimicry to bind TAF-4 (TATA-binding protein associated factor 4), a component of the core promoter recognition complex, through their histone fold domain. This domain resembles the TAF-12 histone fold domain, and in this way OMA-1/-2 prevent the formation of the TAF-4/TAF-12 heterodimer, and sequester TAF-4 to the cytoplasm. The localization of TAF-4 to the cytoplasm prevents RNA polymerase II activation in P0 and P1 (Guven-Ozkan et al. 2008).

At later stages in the germline blastomeres P2-P4 transcription is prevented through PIE-1. PIE-1 is a maternal protein which is present in the nuclei and cytoplasma (low) of the germline blastomeres P0-P4 (Fig.1B) (Mello et al. 1996; Schaner et al. 2003). It is characterized by two predicted RNA-binding domains, TIS11-like zinc fingers, and a specific sequence (YAPMAPT) that resembles the tandem repeats (YSPTSPS) of the carboxy-terminal domain (CTD) of the RNA polymerase II (Batchelder et al. 1999). Transcription initiation and elongation are marked by the phosporylation status of the tandem repeats of the RNA polymerase II. Initiation is marked by Ser5 phosphorylation of the CTD by CDK7, while elongation is marked by Ser5 phosphorylation, plus an additional Ser2 phosphorylation by CDK9/Cyclin T (Phatnani and Greenleaf 2006). A recent study now showed that different sequences within PIE-1 play a role in preventing the CTD phosphorylation of RNA polymerase II. The YAPMAT sequence plus a newly defined sequence (cyclin T binding domain) in PIE-1 are essential for repressing CTD Ser2 phosphorylation in germline blastomeres. PIE-1 is thought to achieve this repression by competing Cyclin T away from the CTD. Surprisingly the deletion of these sequences in PIE-1 leads to ectopic CTD-Ser-2 phosphorylation, while CTD-Ser-5 phosphorylation and transcription are still repressed. Sequences around the YAPMAT motif were found to be critical for suppression of Ser5 phosphorylation, but how PIE-1 suppresses Ser5 phosphorylation is not known (Zhang et al. 2003a; Ghosh and Seydoux 2008). Even if the exact molecular mechanism of PIE-1's function as a transcriptional repressor is not completely defined, the studies on PIE-1 and OMA proteins showed that they function as general repressors of RNA-polymerase II dependent transcription, and that they are required for primordial germ cell formation and to prevent somatic differentiation of germline blastomeres (Mello et al. 1992; Guven-Ozkan et al. 2008).

Consistently it was found that transcriptional regulation is also one of the fundamental mechanisms for PGC specification in the mouse. Loss of Blimp-1, a transcriptional regulator, which is characterized by a SET domain and by Krueppel-type zinc fingers, leads to aberrant gene expression in PGCs (e.g. ectopic expression of homeobox proteins (Hoxa1, Hoxb1)), and to the loss of PGCs during embryogenesis (Ohinata et al. 2005; Vincent et al. 2005). Gene expression profile analysis of developing PGCs showed that genes involved in embryonic development, gastrulation, pattern specification, cell cycle progression, and DNA methylation are down-regulated, while genes for germ cell development (*Blimp-1, fragilis, stella, Dnd1, Kit*) and for transcriptional regulators are up-regulated during PGC specification. Pluripotency genes (*Sox2, Nanog, Zic3*) are initially down-regulated during PGC specification, but start to be expressed from ~E7.0 (Fig.3). This study also showed that the repression of nearly all genes during PGC specification depends on Blimp-1, and that Blimp-1 is also required for the upregulation of several genes necessary for PGC specification, demonstrating Blimp-1's central function in regulating transcription (Kurimoto et al. 2008; Saitou 2009). How exactly Blimp-1 regulates transcription is not understood, however it is involved in the formation of a germ-cell-specific chromatin signature together with the arginine-specific histone methyltransferase, Prmt5 (Ancelin et al. 2006).

Furthermore recently Prdm14, a PR domain-containing transcriptional regulator, which is specifically expressed in PGC from ~E6.5-E13.5, was found to be critical in the regulation of germ line specific chromatin changes and the establishment of pluripotency (Yamaji et al. 2008).

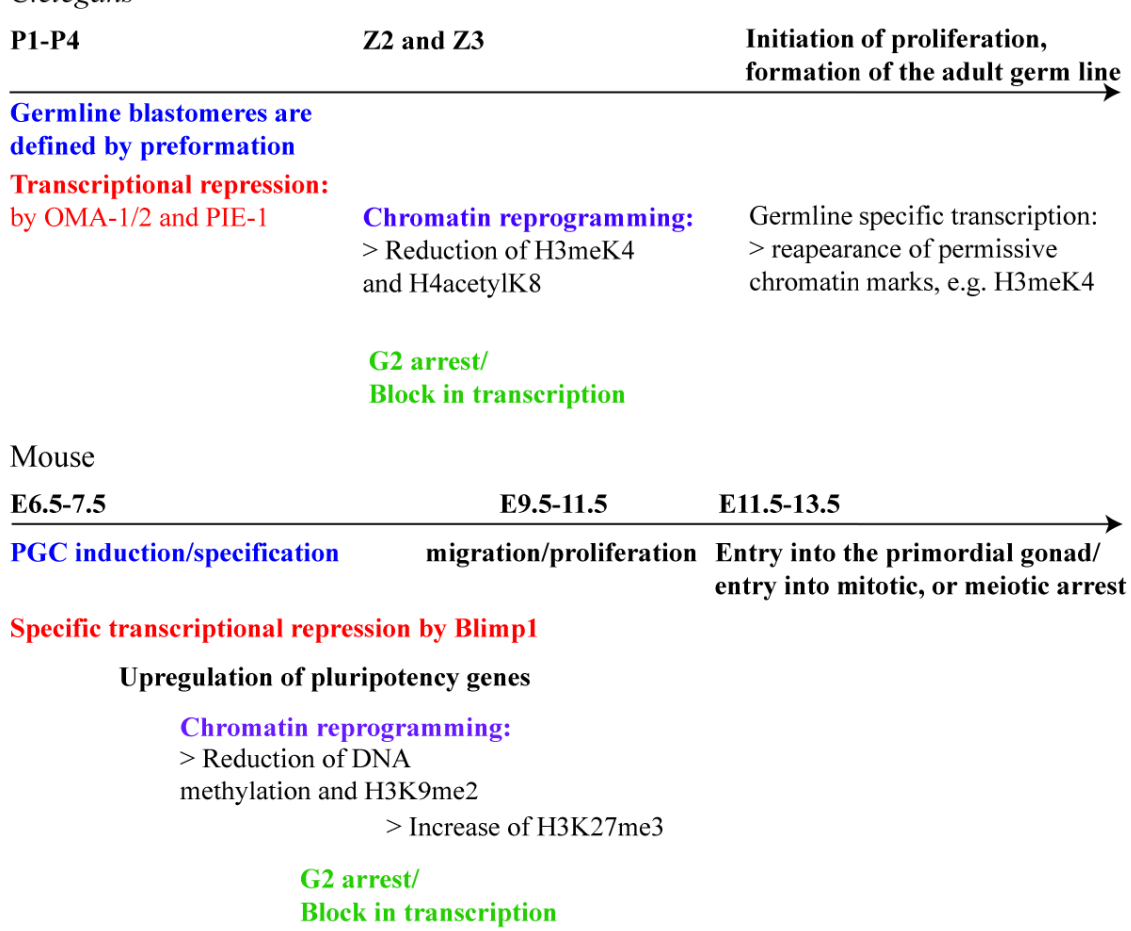
Usually at E 6.75 PGC precursors and soma have an indistinguishable chromatin signature, however at E 8.0, when PGCs start to migrate, DNA methylation, as well as H3K9me2 levels are reduced, and with a delay H3K27me3 levels are increased at E 8.25 (Seki et al. 2005). Modifications of DNA methylation and histone proteins form a complex regulatory network to define a transcriptional repressive or active chromatin. DNA methylation is mainly associated with gene silencing (Li 2002), and the complex pattern of different histone modifications can promote an active, or an inactive chromatin. H3K9me2 and H3K27me3 both promote a transcriptional repressed chromatin (Peterson and Laniel 2004). Hence changes in DNA methylation and histone modifications during PGC development indicate a change in the transcriptional competence of the cells.

Furthermore it is interesting to note that during this transition between different chromatin states from E 8.0-9.5, PGCs pause their global RNA polymerase II dependent

transcription by an yet undefined mechanism and simultaneously enter a G2 arrest (Seki et al. 2007). Eventually this arrest functions to prevent aberrant transcription (Fig.3).

Interestingly global changes in chromatin modifications as well as changes in the cell cycle state are also seen in *C.elegans*. As mentioned before in the germline blastomeres (P1-P4) transcription is regulated through blocking the activity of RNA polymerase II by OMA proteins, and PIE-1. Block in transcription seems to be independent of global changes in chromatin modifications, as germline blastomeres (P1-P4) and the somatic blastomeres both show globally a similar expression pattern of markers for transcriptional permissive chromatin. However simultaneously with the formation of the founder cells of the adult germ line, Z2 and Z3, the mode of transcriptional regulation changes. PIE-1 is lost, and the permissive chromatin modifications, H3meK4 and H4acetylK8, are reduced, and a general chromatin compaction occurs (Schaner et al. 2003).

Fig.3) Transcriptional control is one of the fundamental mechanisms regulating primordial germ cell specification and development.



Similar to the mouse, in *C.elegans* Z2 and Z3 enter a G2 arrest, and the repressive chromatin state is only relieved prior to re-initiation of the cell cycle after hatching (Sulston et al. 1983; Schaner et al. 2003; Fukuyama et al. 2006). The mechanism regulating the G2 arrest is not understood, and only few factors are known to be involved in its regulation. Two conserved putative RNA binding proteins Nos-1 and Nos-2 are required for the maintenance of the cell cycle arrest in Z2 and Z3, as well as in the establishment/maintenance of the repressive chromatin state (Subramaniam and Seydoux 1999; Schaner et al. 2003). Another factor required for the maintenance of the mitotic arrest is DAF-18, the *C.elegans* homolog of the tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome 10) (Fukuyama et al. 2006).

Taken together, these studies show the importance of transcriptional regulating for primordial germ cell specification and development, and importantly these mechanisms are conserved and are also found in other organism, such as *Drosophila* (Nakamura and Seydoux 2008).

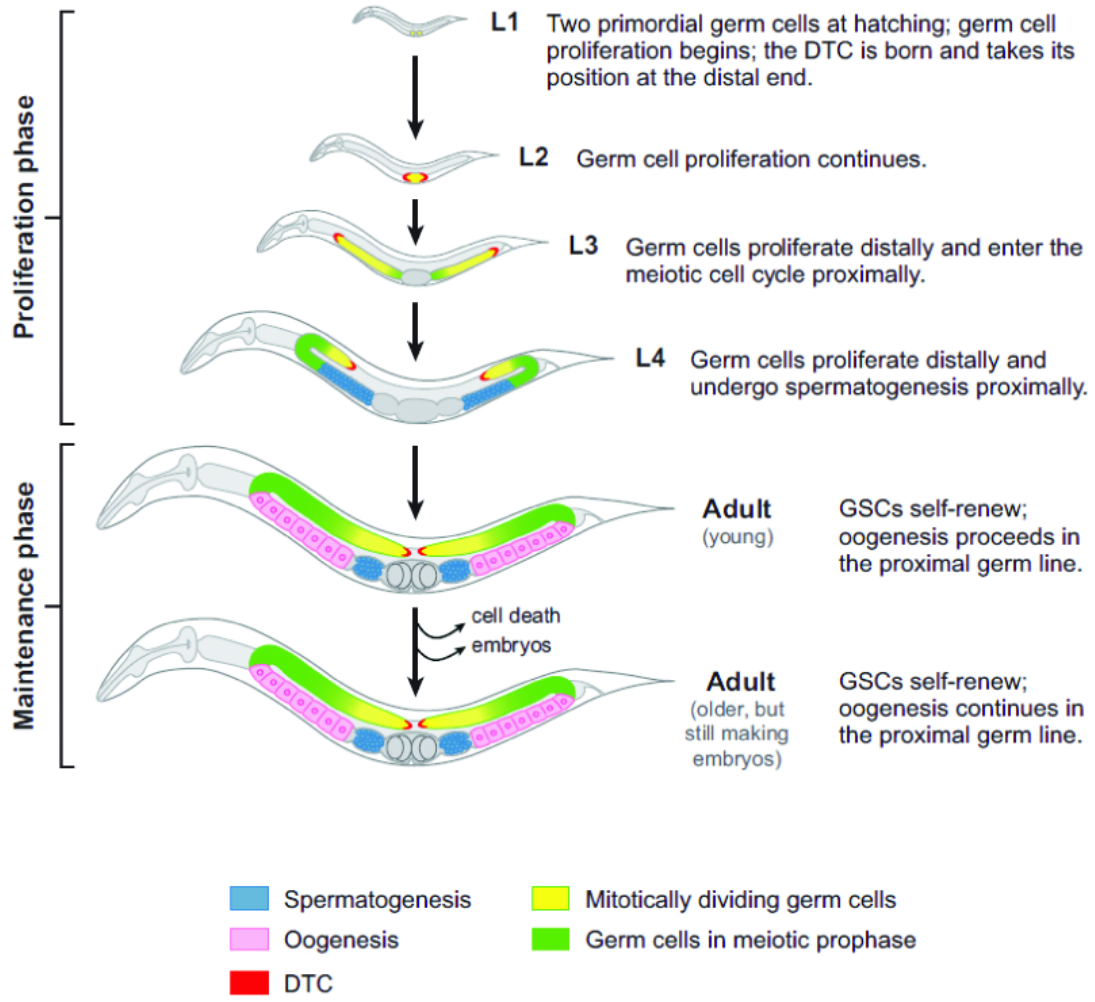
iii. Post-embryonic germ cell development.

1. Building up the germ line in *C.elegans* – from larval stage to adulthood.

In *C.elegans* at hatching in both hermaphrodite and males a gonadal primordium has formed. It is formed by a basement membrane which surrounds 4 cells, the two germline founder cells, Z2 and Z3, and two somatic cells Z1 and Z4, which flank the germline cells. After hatching during larval development, if the nutritional environment is favorable, Z1 and Z4 give rise to the somatic structures of the gonad. In the hermaphrodite they form an anterior and a posterior U-shaped gonad arm, which develop into an ovo-testes, and in males they form a single U-shaped armed testis. In parallel Z2 and Z3 initiate proliferation and build up the population of *C.elegans* germ cells. From the third larval stage on, germ cells in the most proximal region of the gonad enter meiosis and later initiate spermatogenesis, or oogenesis (Fig.4A). This leads to the formation of a distal to proximal polarity in the male and female gonad. In the adult the most distal region (~20 cell diameter) of the gonad contains undifferentiated, mitotically dividing germ cells. Proximal of this region germ cells enter the meiotic S-phase which is followed by the meiotic prophase. Along the proximal direction the germ cells subsequently progress through the different stages of the meiotic prophase and arrest in diakinesis at the most proximal end of the gonad (Fig.4B). The germ

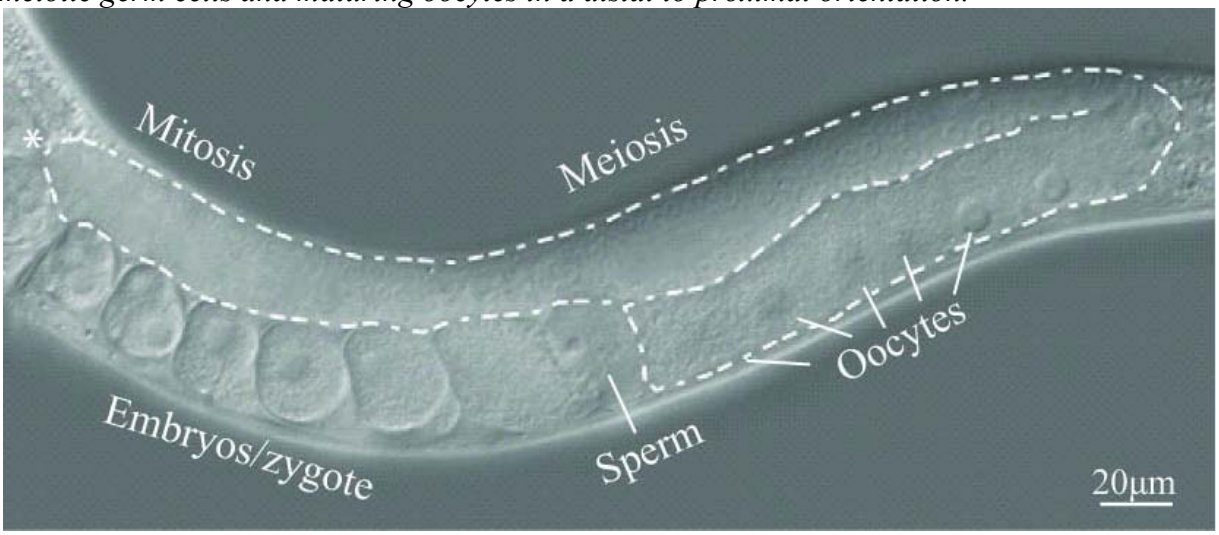
line forms a syncitium, however as great parts of each germline nucleus are surrounded by its own membrane, each germline nucleus and its cytoplasm are referred to as a germ cell (Kimble and Hirsh 1979; Hansen et al. 2004a; Hubbard and Greenstein 2005; Kimble and Crittenden 2007).

Fig.4A) From larval stage to adulthood - establishing the adult germ line.



A) The adult somatic gonad and germ line is built up by four cells, Z1-Z4. Starting form larval stage L1, Z1 and Z4 built up the somatic gonad which harbors the developing germ cells which are formed by Z2 and Z3. The distal tip cell (DTC) of the somatic gonad leads the path of the formation of the U-shaped gonad arms during larvae development and also provides a niche for the mitotically dividing germ cells. During larval stage L3 the most proximal cells enter meiosis, which establishes a distal to proximal orientation in the gonad ((Kimble and Crittenden 2007)).
A and B) (for B, see next page) The adult gonad contains mitotically dividing germ cells in the most distal region (always indicated by an asterisk). Cells in the proximal direction enter and progress through the different stages of the meiotic prophase and undergo gametogenesis in the most proximal end.

Fig.4 B) The adult hermaphrodite gonad harbors mitotically dividing germline stem cells, meiotic germ cells and maturing oocytes in a distal to proximal orientation.



As mentioned before Z2 and Z3 acquire a transcriptionally competent chromatin just before initiation of proliferation after hatching (Schaner et al. 2003). An active chromatin state is maintained in adult germ cells, and germ cells are transcriptionally active during mitosis and the early meiosis (Reinke et al. 2000; Schaner and Kelly 2006). Therefore the cells cannot rely anymore on a global repression of transcription to maintain germline identity, instead they need to use another mechanism.

2. Mechanisms regulating mitosis and meiosis in *C.elegans*.

The mechanisms that initiate proliferation in Z2 and Z3 are not known. However proliferation is stimulated through the distal tip cell (DTC) of the somatic gonad, which forms a niche for mitotically dividing germ cells. The depletion of the DTC leads to an arrest of germ cell proliferation and premature entry into meiosis (Kimble and White 1981). This phenotype is exactly copied by a mutation in the gene *glp-1*/Notch (Austin and Kimble 1987). It was found that the DTC promotes germline proliferation/self-renewal through the expression of the two GLP-1 ligands, LAG-2 and APX-1, while GLP-1 is expressed by the germ cells in the distal region of the gonad (Henderson et al. 1994; Nadarajan et al. 2009). Glp-1/Notch signaling is absolutely required to induce proliferative growth and to maintain the germ line throughout development. In addition other factors were found to be required for post-embryonic germline proliferation. Loss of *glp-4*, whose molecular identity is not defined, prevents germline formation. In *glp-4 (-)* animals only a small number of germ cells are formed, and these cells arrest in the mitotic prophase (Beanan and Strome 1992). In

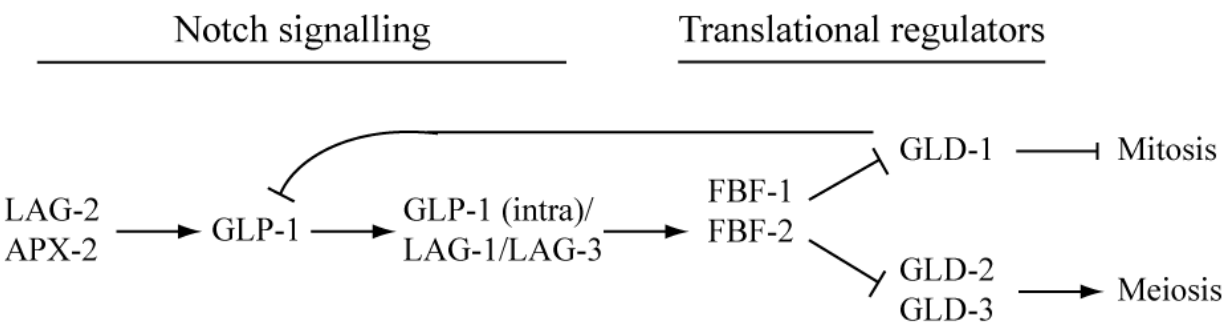
addition loss of the maternally provided factors, MES-2, MES-3, and MES-4, which belong to the polycomb group proteins and function in a complex, severely impairs the establishment of the germ line (Capowski et al. 1991). The MES complex is required for H3K27 tri-methylation in the PGCs, and for the H3K27 di- and tri-methylation in mitotic and early meiotic adult germline cells (Bender et al. 2004). However the molecular mechanism leading to germ line degradation and sterility in the *mes* mutants is not understood.

Starting from the larvae stage L3 germ cells in the proximal region enter meiosis. The molecular mechanisms regulating the mitosis/ meiosis decision in *C.elegans* were extensively studied in the adult germ line. As described before GLP-1/Notch promotes mitosis in distally located undifferentiated germline cells. By genetic and molecular experiments, the major components of the GLP-1/Notch signaling pathway are well described. The canonical Notch signaling pathway relies on a conserved pathway with four core components: the DSL ligand (for Delta, Serrate, and LAG-2), LNG receptor (for LIN-12, Notch, and GLP-1), the CSL transcription factor (for CBF-1 Su(H), and LAG-1), and the MAML transcriptional coactivator (Mastermind-LAG-3). The interaction between GLP-1/Notch and its ligands (LAG-2 and APX-2) is thought to lead to a cleavage step that liberates the intracellular part of GLP-1, GLP-1(Intra). GLP-1(Intra) then translocates to the nucleus, where it forms a ternary complex with LAG-1/CSL, a transcription factor, and LAG-3, a coactivator. This binding leads to a conversion of LAG-1 from a repressor to a transcriptional activator (Fig.5) (Kimble and Simpson 1997; Greenwald 2005; Hansen and Schedl 2006). However how precisely GLP-1 signalling promotes mitosis and represses meiosis is only very poorly understood, as only two direct targets of this pathway are defined, *lip-1* and *fbf-2*. LAG-3 was found to co-immunoprecipitate with the lip-1 promoter and LAG-1 was found to bind to consensus binding sites within the 5' region of *fbf-2* in vitro (Lamont et al. 2004; Lee et al. 2006).

The link between GLP-1/Notch signaling and RNA binding proteins that regulate mitosis and meiosis was made through this finding that the *fbf-2* 5' flanking region contains 4 LAG-1 binding sites and its expression is positively regulated by GLP-1 signaling (Lamont et al. 2004). FBF-2, as well as FBF-1, two nearly identical regulators, belong to the Pumilio and FBF (PUF) protein family. They are characterized by 8 RNA binding PUF-repeats (Zhang et al. 1997; Wickens et al. 2002). Both FBF proteins act redundantly in the distal germ line, but they have different functions in fine tuning the mitosis/meiosis decision (Lamont et al. 2004). FBF-1 and FBF-2 both bind the same FBF binding element (FBE) (Bernstein et al. 2005),

and by genetic experiments FBFs were defined to regulate various proteins that are involved in the mitosis/meiosis, and the spermatogenesis/oogenesis decision, (FEM-3, GLD-1, GLD-3, LIP-1, FBF-1/FBF-2, MPK-1) (Zhang et al. 1997; Crittenden et al. 2002; Eckmann et al. 2004; Lamont et al. 2004; Lee et al. 2006; Lee et al. 2007). Recent experiments suggest that FBF proteins can have a dual function in promoting, as well as repressing protein expression. FBF proteins bind specifically the *gld-1* 3'UTR through FBEs (Crittenden et al. 2002; Suh et al. 2009), but depending on the fate of the germ cells (spermatogenic, or oogenic mode of germ cell development) FBFs repress, or promote GLD-1 expression. In an oogenic germ line loss of FBF-1 and FBF-2 leads to ectopic GLD-1 expression in the distal gonad. In vitro experiments showed that FBFs interact with CCF-1, a Pop2p class deadenylase, and promotes its activity, suggesting that FBFs prevent protein expression in the distal region through mRNA deadenylation. However in a spermatogenic germ line the loss of FBF-1 and FBF-2 leads to reduced GLD-1 expression in the transition zone. In vitro experiments and immunoprecipitation showed that FBF-1 forms a complex with GLD-2, a poly-A polymerase, and GLD-3/Caudal. In vitro experiments also showed that FBF-1 enhances the poly-a polymerase activity of GLD-2, leading to the hypothesis that FBFs promote protein expression through polyadenylation of the mRNA 3'UTR (Suh et al. 2009). In this way FBFs regulate and are centered between the major pro-mitotic pathway, GLP-1/Notch signaling, and the two major pro-meiotic pathways formed by GLD-1 and GLD-2 (Fig.5).

Fig.5) The regulatory network of the mitosis/meiosis decision in the adult C.elegans hermaphrodite germ line.



GLD-1, a member of the STAR (signal transduction and activation of RNA) family of RNA-binding proteins (Vernet and Artzt 1997), is expressed in the cytoplasm of early meiotic germ cells in the transition zone (leptotene, zygotene) till the bend region of the gonad, where cells exit pachytene (Jones et al. 1996). In *gld-1(-)* animals germ cells are able

to enter meiosis, due to redundant pro-meiotic pathways, however germ cells fail to progress through meiosis, and instead re-enter mitosis forming a germline tumor (Francis et al. 1995a; Kadyk and Kimble 1998). In the *C.elegans* germline GLD-1 functions as a translational repressor, and while several targets, which are involved in the spermatogenesis/ oogenesis decision, oocyte maturation, embryogenesis, or DNA damaged induced apoptosis (e.g. TRA-2, RME-2, PAL-1/Caudal, MEX-3, CEP-1/p53) were defined (Jan et al. 1999; Lee and Schedl 2001; Mootz et al. 2004; Schumacher et al. 2005), GLD-1's function in regulating entry into and progression through meiosis is only partially understood. Interestingly GLD-1 prevents GLP-1 repression through translational repression of *glp-1* mRNA in proximal cells of the distal region (Marin and Evans 2003). However loss of GLP-1 repression in *gld-1(-)* animals can only partially explain tumor formation, as in the *gld-1, glp-1* knock out animals germ cells still enter meiosis, fail to progress through meiosis and re-enter mitosis forming a germline tumor (Francis et al. 1995b). Despite the fact that the *gld-1, glp-1* germline tumor is smaller this shows that additional factors need to be repressed to prevent tumor formation and to allow progression through meiosis.

Similar to the *gld-1(-)* phenotype, germ cells in *gld-2(-)* animals fail to progress through meiosis, and to a lesser extent as in *gld-1(-)* mutant, re-enter mitosis (Kadyk and Kimble 1998). GLD-2 shows strongest expression in the cytoplasm of pachytene cells and oocytes, and functions as a cytoplasmic poly(A) polymerase, which promotes meiosis through polyadenylation and activation of *gld-1* mRNA (Wang et al. 2002; Suh et al. 2006). However additional undefined pro-meiotic GLD-2 targets must exist, as germ cells in the *gld-1* mutant are still able to enter meiosis.

Additional factors exist to promote GLD-1 and GLD-2 activity. GLD-3, which belongs to the Bicaudal-C family of RNA binding proteins interacts with GLD-2 and is thought to confer RNA binding affinity to the GLD-2/GLD-3 complex, as GLD-2 misses a RRM (RNA recognition motif)-like domain (Wang et al. 2002). NOS-3, a member of the conserved Nanos family of RNA-binding proteins, shows functional redundancy with GLD-2 in promoting GLD-1 expression through an undefined mechanism (Hansen et al. 2004b).

In addition to the three major pathways built by GLP-1, GLD-1, and GLD-2, further factors are involved and play an underlying function within this network. The putative RNA-directed RNA polymerase (RdRP) EGO-1 has a subtle function in promoting mitosis over meiosis in parallel to GLP-1 signaling (Vought et al. 2005). EGO-1's molecular function is not defined; however EGO-1 activity is known to affect nuclear pore complex (NPC) and P-

granule formation (Vought et al. 2005), and it is required for H3HK9me2 on unpaired chromosomes during meiosis (Maine et al. 2005).

Many more translational RNA binding proteins were identified to regulate mitosis/meiosis, such as PUF-8 and MEX-3. Both are expressed in the distal region of the gonad and seem to function in parallel to GLP-1 in promoting mitosis (Ciosk et al. 2004; Ariz et al. 2009). PUF-8 is a member of the PUF family of translational regulators, and its loss leads to germline tumor formation, as primary spermatocytes fail to progress through meiosis and re-enter mitosis (Subramaniam and Seydoux 2003). However neither the molecular function of PUF-8, nor its targets are defined. MEX-3, a KH domain protein, is expressed in the distal gonad, the proximal gonad, and in early embryonic blastomeres (Draper et al. 1996). MEX-3 is thought to function as a translational regulator and regulates the expression of PAL-1 in the early embryo (Hunter and Kenyon 1996). Another MEX-3 target, RME-2, a yolk sac protein, becomes ectopically expressed in the distal region of *mex-3 (-)* gonads, but otherwise *mex-3 (-)* gonads have no obvious germline defects (Ciosk et al. 2004). Another example is FOG-1, which belongs to the CPEB (Cytoplasmic Polyadenylation Elements) family of RNA regulatory proteins, and which regulates mitosis downstream of GLP-1 (Thompson et al. 2005). An additional RNA binding protein that regulates progression through meiosis is DAZ-1. DAZ-1 belongs to the conserved DAZ (deleted in azoospermia) family, which consists of DAZ, DAZL and BOULE (Reijo et al. 1995; Cooke et al. 1996; Eberhart et al. 1996). The family members are characterized by a conserved RNA recognition motif (RRM) and at least one copy of a DAZ motif, which has been implicated in protein-protein interactions. Loss of DAZ-1 in *C.elegans* leads to a meiotic arrest during prophase, and seems to function downstream of GLD-1 (Karashima et al. 2000).

Furthermore regulatory factors were defined to influence translational in the gonad, such as ATX-2/Ataxin 2, which plays a role in regulating the function of MEX-3 and GLD-1 (Ciosk et al. 2004).

Genetic experiments showed the involvement of several more RNA binding proteins in the mitosis/meiosis decision, and interestingly most of the described regulators are also involved in the regulatory network of the sperm/oocyte decision. Altogether these studies show how much the adult *C.elegans* germ line relies on translational regulation as a major mechanism to regulate the germ cell development in larvae and adults (Hansen and Schedl 2006; Kimble and Crittenden 2007).

3. Conserved RNA regulators play a fundamental role in mouse germ cell development.

The network of factors regulating germ cell development in the mouse is only very poorly understood. However also in the mouse various post-transcriptional regulators were found to have a fundamental role in regulating protein expression during germ cell development. Many of the protein families being involved in the *C.elegans* germline development are also critical regulators during mouse germ cell development and will be discussed in the following paragraph. However these factors often regulate different stages of germ cell development in the mouse and no functional network of RNA regulators could be defined so far.

Similar to the loss of *daz-1* in *C.elegans*, germ cells in *Dazl (-)* mouse show no mitotic defects but fail to progress through meiosis in both sexes (Saunders et al. 2003). DAZ proteins seem to function as translational activators, as ectopically expressed members of the human and mouse DAZ family are able to initiate translation of a reporter mRNA in *Xenopus laevis* oocytes, and to interact with poly (A)-binding proteins PABPs (Collier et al. 2005). In mouse testis expression of mouse vasa homologue (Mvh) and the synaptonemal complex protein, SYCP3, is regulated by translational activation through DAZL. Importantly loss of either Mvh or SYCP3 leads to a block during early meiotic prophase, which is similar to the phenotype seen in *Dazl (-)* animals (Tanaka et al. 2000; Yuan et al. 2000; Saunders et al. 2003). Human DAZ and DAZL interact with human Pumilio-2 (Moore et al. 2003), which links them to the conserved family of PUF proteins, which not only in *C.elegans* are critical regulators of germ cell development. A common set of RNA targets for DAZL and Pum2 were found (Fox et al. 2005), however the function of Pumilio proteins (Pum1, Pum2) in mouse germ cells still needs to be defined (Xu et al. 2007).

Three homologs of the zinc-finger RNA-binding protein Nanos have been defined in the mouse. *nanos-1* mRNA is not expressed in developing germ cells and *nanos-1(-)* animals are viable, show no significant abnormality, and are fertile (Haraguchi et al. 2003). *nanos2* mRNA is initially maternally provided but then it is mainly expressed in male germ cells (TP 13.5-16.5). *nanos3* mRNA is expressed in migrating germ cells (till E 13.5d). Absence of *nanos2* leads to loss of all male germ cells. Germ cells are normally localized in the testicular cord, but starting from E 15.5 germ cells become localized outside the seminiferous tubules and seem to enter apotosis. Interestingly Nanos2 levels are adjusted through translational regulation mediated by the 3'UTR, which is important for early spermatogensis. On the other

hand the female *nanos2 (-)* gonads are morphologically normal and female mice are fertile (Tsuda et al. 2003; Tsuda et al. 2006). In contrast *nanos3 (-)* animals show defects in the male and female germ line. PGC formation occurs normally but cells fail to proliferate and enter apotosis during the migration phase (Tsuda et al. 2003; Suzuki et al. 2008). Consistently with this phenotype, Nanos-3 is expressed during PGC specification and migration (E7.25 till E13.5), and is downregulated with the meiotic entry in female germ cells, while it is weakly expressed during the mitotic arrest of male germ cells and is strongly expressed in spermatogonia, the adult sperm stem cells. Interestingly Nanos3 co-localizes with components of stress granules and processing bodies, implicating a role of Nanos3 in translational control in mouse PGCs (Yamaji et al. 2009).

An ortholog of GLD-1, SAM68, which belongs to the STAR family (Vernet and Artzt 1997), is involved in various processes of RNA metabolism, such as nuclear export (Li et al. 2002a), or alternative splicing (Paronetto et al. 2007; Chawla et al. 2009). Besides its occurrence in somatic cells, SAM68 is expressed during oocyte maturation and early embryogenesis. In the zygote and during early embryogenesis SAM68 co-localizes with components of the translational initiation complex and hence might have a role in translational regulation of maternal mRNAs (Paronetto et al. 2008). In males SAM68 is expressed in the nucleus of spermatogonia, the sperm stem cells. During early meiotic prophase SAM68 is initially downregulated, but becomes expressed again in the nuclei of pachytene spermatocytes. During meiosis SAM68 localizes to the cytoplasm of secondary spermatocytes (finished second meiotic division) and round spermatides (immature sperm) (Paronetto et al. 2008; Paronetto et al. 2009). In spermatocytes SAM68 binds the translational initiation complex, associates with polysomes and is required for the translation of defined SAM68 targets, showing that SAM68 function as a translational regulator in male germ cells. Interestingly members of the STAR family can integrate signal transduction pathways and RNA metabolism. In addition to its RNA binding domain SAM68 contains several sequences that are potential binding sites for various kinases and it was found that the phosphorylation status of SAM68 affects its cellular location or its splicing activity (Vernet and Artzt 1997; Matter et al. 2002; Lukong and Richard 2003; Lukong et al. 2005). In spermatocytes phosphorylation of SAM68 by the kinases, ERK1/2, correlates with its localization to the cytoplasm, its association with the translation initiation complex and the translation of SAM68 targets (Paronetto et al. 2009). This shows that SAM68 functions between signaling and RNA metabolism in germ cells.

Taken together while the advantages of a model system like *C.elegans* allowed the analysis of a complex network of regulators governing germ cell development, and revealed the importance of RNA regulation, we have only a poor understanding of this network in the mouse. However recent findings show the importance of RNA regulation during mouse germ cell development and the identification of conserved factors in various systems will help to further define the mechanism regulating germ cell development in the mouse.

iv. RNA regulation, a conserved mechanism to maintain the underlying totipotency in germ cells.

Interestingly, as described before, in both organisms, the mouse and *C.elegans,* RNA regulators are critical for germ cell development, and recent findings showed that they are also required to maintain germline identity and to prevent germ line to soma transition. In the mouse the loss of DND-1, a RRM (RNA recognition motif) protein, and in *C.elegans* the loss of GLD-1, a STAR protein, lead to the formation of unusual germline tumors called teratoma (Youngren et al. 2005; Ciosk et al. 2006; Cook et al. 2009).

Teratoma formation can be studied in male 129Sv/J Ter ($Dnd^{ter/ter}$) mice which show a very high incidence of testicular teratoma, while in female 129Sv/J Ter ($Dnd^{ter/ter}$) mice PGCs are lost early in development (Stevens 1973; Youngren et al. 2005). The testicular teratoma originates from PGCs at E12.5 (Stevens 1962), however the molecular mechanism leading to teratoma formation is not known. Only the presence of an RRM (RNA recognition motif) motif in DND1 suggests that RNA regulation plays a role in teratoma formation (Youngren et al. 2005).

Recently a finding by Dr.Rafal Ciosk showed that in *C.elegans* the loss of the translational regulators GLD-1 and MEX-3 not only lead to the formation of germline tumors, but also to germ line to soma transition, which we refer to as transdifferentiation (TD). The germline tumors of the *gld-1,* and *mex-3, gld-1*mutants, consist of a heterogeneous population of mitotic, meiotic, and necrotic cells, as well as postmeiotic cells that lost germline identity and differentiated into various types of somatic cells (e.g. muscle, neurons, intestinal cells) (Fig.6). This phenotype is reminiscent to the mouse and human teratoma and hence we refer to it as the worm teratoma. This finding showed that also germ cells in *C.elegans* have an underlying totipotency, and it clearly showed the importance of RNA regulation in maintaining germline identity and totipotency. Importantly this work established

C.elegans as a genetically tractable model system to study the mechanisms that are required to maintain germ cell fate and that promote germ line to soma transition (Ciosk et al. 2006).

Fig.6) The worm teratoma.

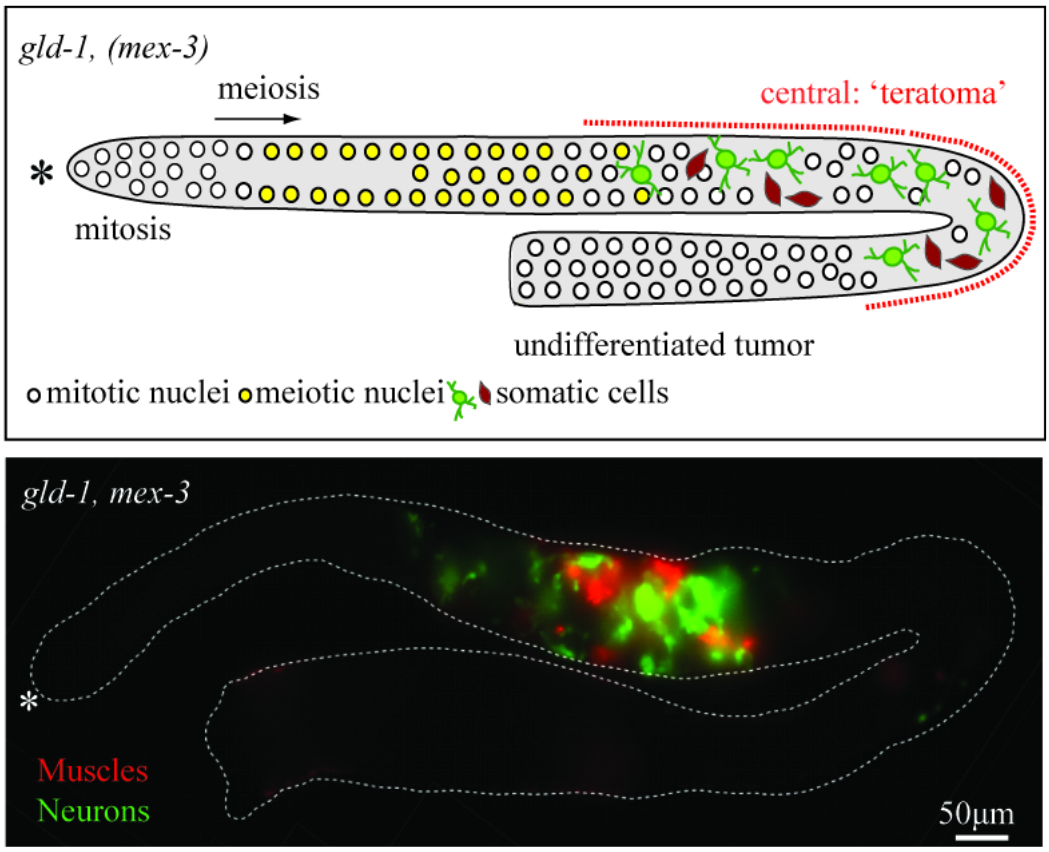


Fig.6) Germ cells in the *gld-1*, or *gld-1,mex-3* mutant fail to progress through meiosis, and instead re-enter mitosis forming a germline tumor. Interestingly within the central region of the gonad terminally differentiated somatic cells, such as muscles (red) and neurons (green), are formed. This phenotype is reminiscent to an unusual mammalian germline tumor, called teratoma. Therefore germ line to soma transition in the *C.elegans* gonad is referred to as the worm teratoma (Ciosk et al, Science 2006).

b. Open Questions

i. How do germ cells achieve the contradictory task of differentiating into highly specialized cells, while maintaining an underlying totipotency?

Germ cells develop into highly specialized cells, oocytes and sperm. After fertilization these cells form a totipotent cell which is able to generate the whole organism. Germ cells per se have an underlying totipotency, which becomes obvious in pluripotent cell lines that can be derived from various germ cells, such as PGCs, or spermatogonial stem cells in the mouse, or in their ability to form teratomas in mouse and *C.elegans*. However the mechanisms that maintain germ cell identity and prevent teratoma formation are not understood neither in the mouse, nor in *C.elegans*. Therefore we used *C.elegans* as a model system to ask what the mechanisms are that promote germ cell development and prevent germ line tumor formation. Further we used teratoma formation in *C.elegans* as a tool to study the mechanisms that promote germ line to soma transition.

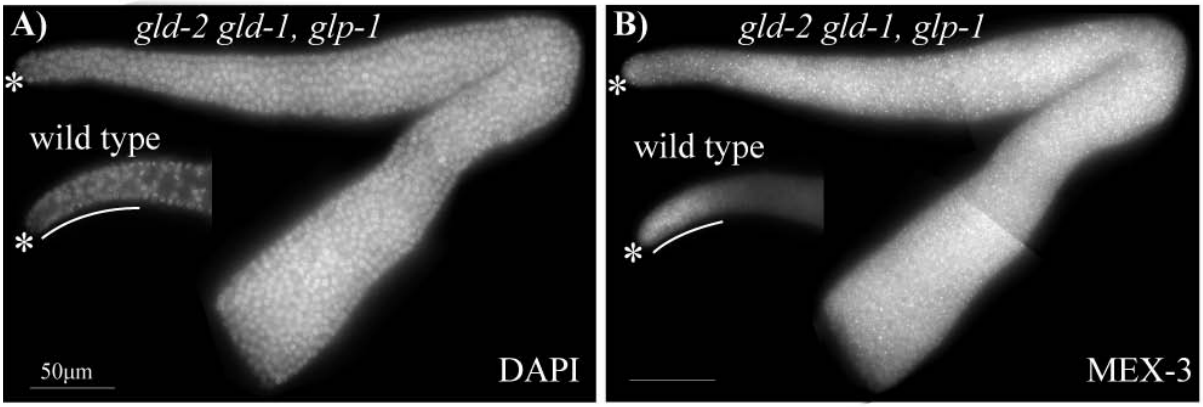
To address these questions we first decided to characterize the different stages which lead to germ line to soma transition; second we defined GLD-1 targets, as already loss of this translational regulator leads to germ line to soma transition; and finally genetic analysis of GLD-1 targets was used to define the underlying molecular pathways.

2) RESULTS

a. Germ line to soma transition is an orderly, multistep process.

The great heterogeneity in the *mex-3, gld-1* germline tumor makes it very difficult to reveal the origin of teratoma formation (Ciosk et al. 2006). Therefore we decided to analyze a genetic background in which the major regulators for mitosis (GLP-1), and meiosis (GLD-1, and GLD-2) were knocked out. The *gld-2 (q497), gld-1 (q485), glp-1(q175)* mutant (triple mutant) gonad lacks polarity for any specific germ cell fate. Germ cells in the triple mutant show defects in entering meiosis, and form a germline tumor consisting of only proliferating cells (Hansen et al. 2004a). Phenotypically by differential interference contrast (DIC) microscopy and by DAPI staining these proliferating cells resemble undifferentiated wild type germline stem cells (data not shown, Fig.7A). We found that like wild type germline stem cells in the distal region, all proliferating cells in the triple tumor showed MEX-3 expression (Ciosk et al., 2004) (Fig.7B), setting up a perfect system to investigate the function of MEX-3.

Fig.7 The germline tumor in the gld-2 gld-1, glp-1 mutant is formed by a homogenous population of MEX-3 positive germline "stem cell like" cells.



A and B) Shown are gonads of young adult *gld-2 gld-1, glp-1* and wild type (wt) animals. The partial wt gonad shows the mitotic region (indicated by the white line) and the meiotic transition zone (leptotene, zygotene stage). The gonads were stained with DAPI (A) and MEX-3 (B).

Knock down of MEX-3 by RNAi or knock out of MEX-3 in the *gld-2 (q497) gld-1 (q485) mex-3 (or20), glp-1(q175)* quadruple mutant leads to teratoma formation, which could be detected in extreme cases throughout the whole gonad (distal to proximal end). Two days after the L4/adult molt (TP2) we could detect muscles in 50 % (n=50), or neuronal reporter expression in 83% (n=35) of the gonads (Fig.8, data not shown).

Fig.8 MEX-3 prevents germ line to soma transition in the gld-2 gld-1, glp-1 mutant.

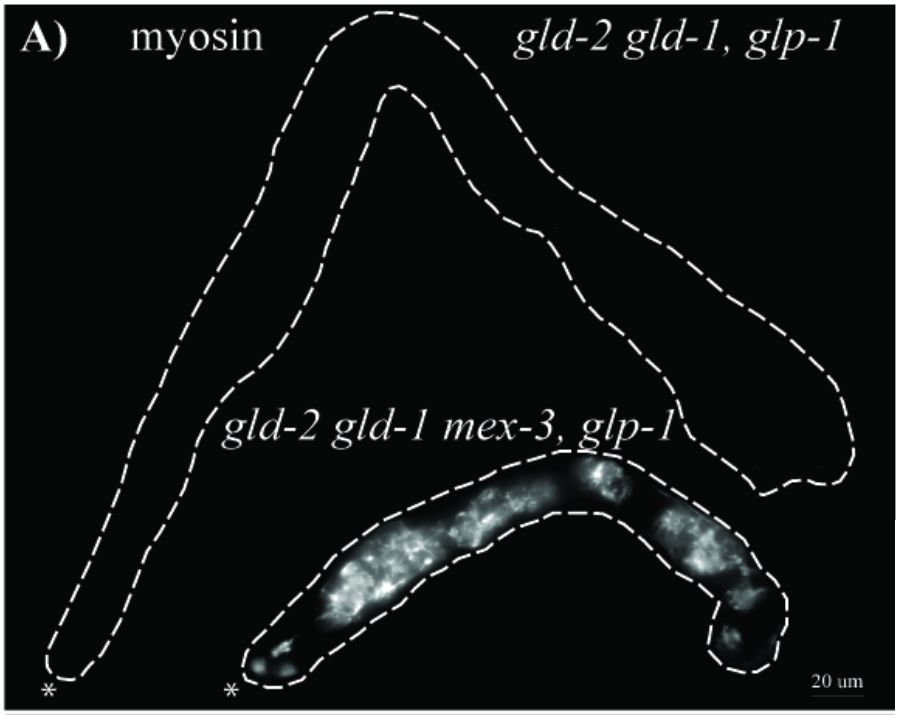
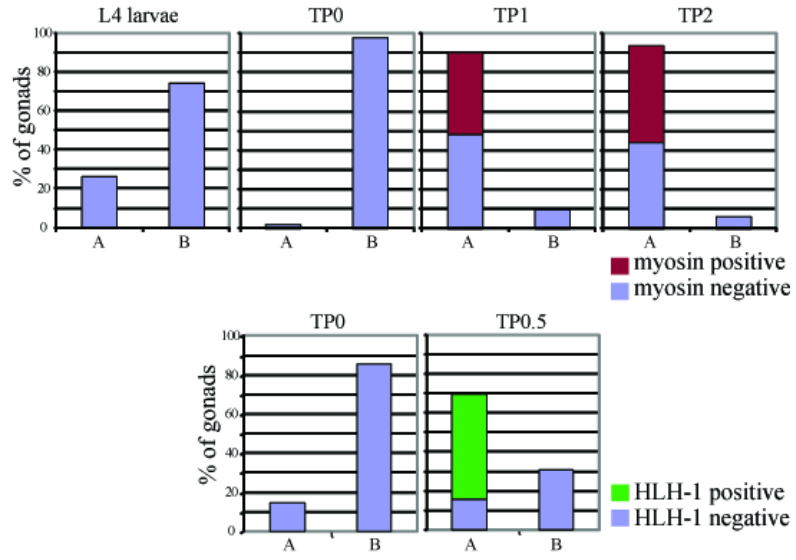


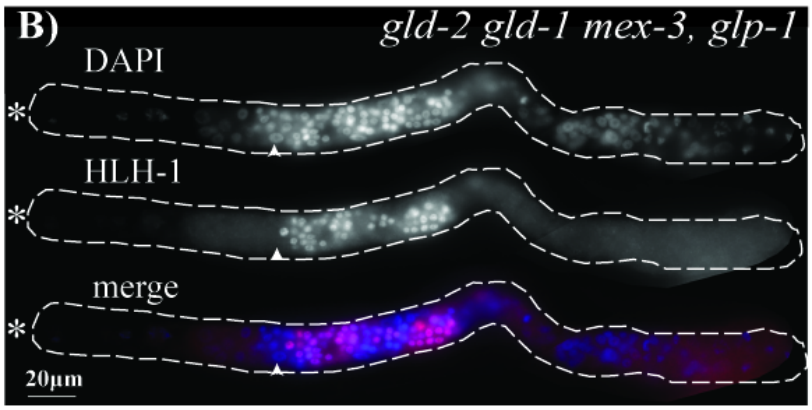
Fig.8) At time point TP2 gonads of the indicated genotype were stained for muscle myosin to visualize teratoma formation.

We also noticed that at this time point the gonads showed a variable degree in size and cell number. It ranged from small gonads that contained only few, manly necrotic cells, to big gonads that contained very many proliferating cells. To monitor teratoma formation, we performed a time course experiment. We found that germ cells go through different phases according to their appearance by DAPI staining. At L4 stage 26% of the gonads contained cells with a nuclei size of ~4µm, while 73% of the gonads contained only cells with an increased nuclei size of >5.0µm and de-condensed chromatin. Later at the L4/adult molt (TP0) 98% of the gonads contained only cells having a nuclei size of ~5.5µm. This changed again at TP1, at which 91% of the gonads contained cells having a nuclei size of only ~4µm. This time course experiment suggested to us that initially up to the L4 stage germ cells undergo proliferation (small nuclei), then at TP0 cells arrest and de-condense (increased nuclei size), and later some cells re-enter proliferation (small nuclei) and form a tumor. Interestingly only in gonads that contained cells with small nuclei, we could detect muscle formation (Fig.9A). Furthermore HLH-1/MYOD, a transcription factor required in muscle formation (Chen et al. 1994), could only be detected in cells having a small nuclei at TP0.5, but not in cells having a big nuclei (Fig.9B), or at TP0 (Fig.9A), suggesting that re-entry into mitosis and proliferation precedes transdifferentiation.

Fig.9) Re-entry into proliferation precedes transdifferentiation.

A) gld-2 gld-1 mex-3, glp-1





A) Cells in the *mex-3 gld-2 gld-1, glp-1* mutant enter an apparent arrest phase and re-enter proliferation before transdifferentiation. In a time course experiment gonads were defined into two categories by the appearance of the nuclei in the DAPI staining. Gonads that contained cells with small nuclei (~4µm) were defined as proliferating (=A), and gonads that only contained cells with increased nuclei size (>5.0µm) were defined as arrested (= B). The graphs show the percentage of gonads which were positively or negatively stained for the muscle markers myosin and HLH-1, respectively.
B) Germ line to soma transition can only be detected in cells which have small nuclei. Gonads were stained for HLH-1 at TP0.5. HLH-1 positive cells have small nuclei, while cells that have large, decondensed nuclei are negative (arrow head).

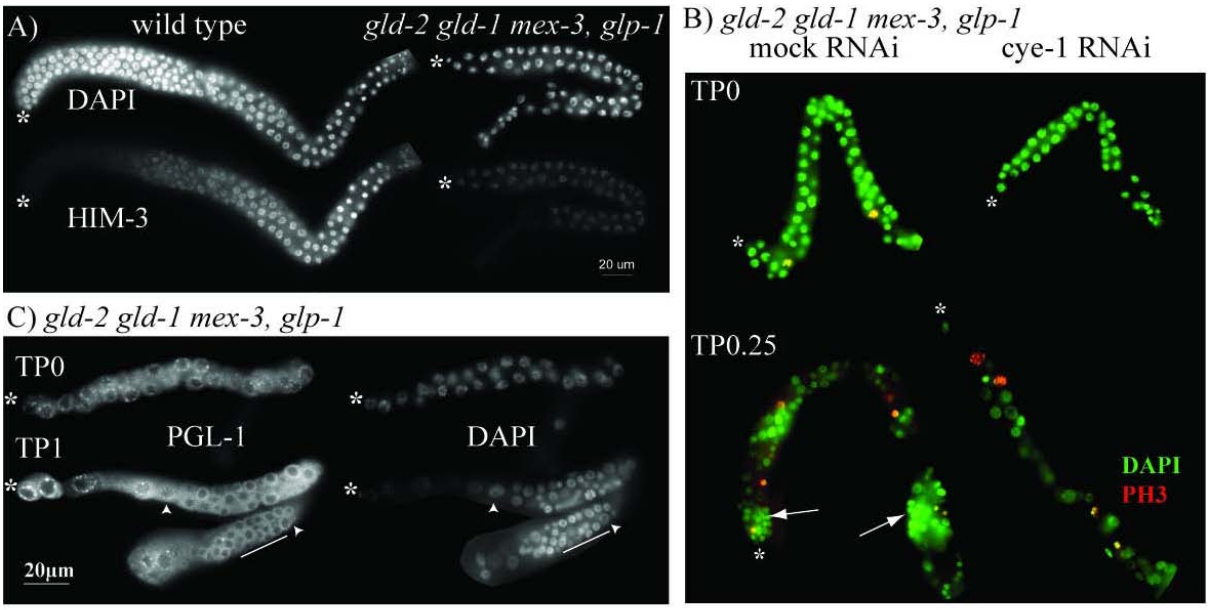
To define the cell cycle stages germ cells go through before transdifferentiation, we stained for the meiosis specific axis component HIM-3 (Zetka et al. 1999). This showed that cells at TP0, which had de-condensed chromatin and a big nucleus, had loaded HIM-3 onto the chromatin, showing that these cells execute at least some aspects of meiosis (Fig.10A).

Consistent with a later exit of meiosis and re-entry into mitosis, we could detect increasing numbers of Histone H3-Ser-10-P (PH3) positive cells, a marker for chromatin condensation (Hsu et al. 2000). On average a gonad contained 2.9 (+/- 1.1), 11.6 (+/- 2.7) PH3 positive cells at time points 0, 0.25, respectively (Fig.10B, left gonads).

To address the question whether re-entry into mitosis also marks the time point of germ line to soma transition, we stained for PGL-1 a component of germline specific structures, called P-granules (Kawasaki et al. 1998). At TP0 we could detect PGL-1 granules attached to the nuclear envelope of arrested cells (big nuclei) in all gonads. However often

these cells showed an abnormal appearance of P-granules, e.g. less P-granules attached to the nuclei, or diffuse staining, instead of a clear spots. At TP1 arrested cells still maintained P-granules, while the majority of cells that had re-entered proliferation were negative for P-granules. However we could observe proliferating cells (small nuclei) which had P-granules, leading to the interpretation, that the loss of P-granules is not an active process, instead P-granules are lost during proliferation (Fig.10c).

Fig.10) Germ cells in the gld-2 gld-1 mex-3, glp-1 mutant fail to progress through meiosis, and re-enter proliferation. This leads to loss of germline identity.

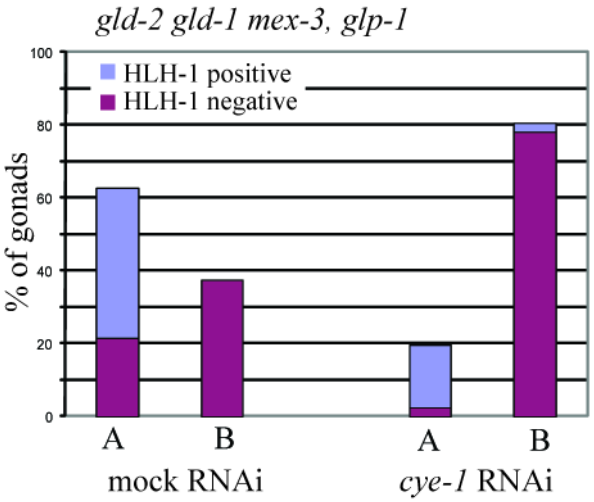


A) Cells in the *gld-2 gld-1 mex-3, glp-1* mutant enter meiosis. At TP0 wt and *gld-2 gld-1 mex-3, glp-1* gonads were stained for the meiosis marker HIM-3. In wild type gonads HIM-3 is expressed as soon as cells enter the meiotic prophase, in the quadruple mutant HIM-3 is expressed throughout the gonad.
B) Cells in the *gld-2 gld-1 mex-3, glp-1* mutant re-enter mitosis. At TP0 and TP0.25 gonads of mock and *cye-1* RNAi treated animals were stained for the M-phase marker H3-Ser-10-P. Starting from TP0 an increasing number of cells entering mitosis (PH3 positive) can be detected in mock treated animals. Entry into mitosis and proliferation was greatly reduced in *cye-1* RNAi treated animals. Arrows points to cluster of proliferating cells in mock treated animals at TP0.25.
C) Proliferating cells lose germ cell identity. Gonads of *gld-2 gld-1 mex-3, glp-1* animals were stained for the germline specif P-granule component PGL-1. Arrow heads indicate arrested cells (big nuclei) which maintain P-granules, while the line indicates a region of proliferating cells (small nuclei), which have lost or have weak P-granules.

The observation that proliferation precedes teratoma formation let us ask whether re-entry into proliferation is required for germ line to soma transition. We found that the depletion of the cell cycle factor CYE-1/Cyclin E greatly reduces the extent of proliferation. Knock down of *cye-1* impaired re-entry into mitosis, as the number of PH3 positive cells was greatly reduced at TP0.25 (2.4 (+/- 0.8) in *cye-1* RNAi animals compared to 11.6 (+/- 2.7) in

mock treated animals), and moreover an average gonad of mock treated animals contained several clusters of proliferating cells, which could not be observed in *gld-2 gld-1 mex-3, glp-1, cye-1* RNAi animals (Fig.10B, arrows). Depletion of CYE-1 prevented proliferation, and not only delayed the cell cycle, as at the late TP2.25 80% of the gonads only contained arrested cells (data not shown). Preventing proliferation also greatly reduced the rate of germ line to soma transition. In mock treated animals 42% (n=56) showed HLH-1 expression in the gonad, but in CYE-1 depleted gonads only 19% had HLH-1 positive cells. Tellingly of these 19% of gonads, 17% contained proliferating cells (Fig.11).

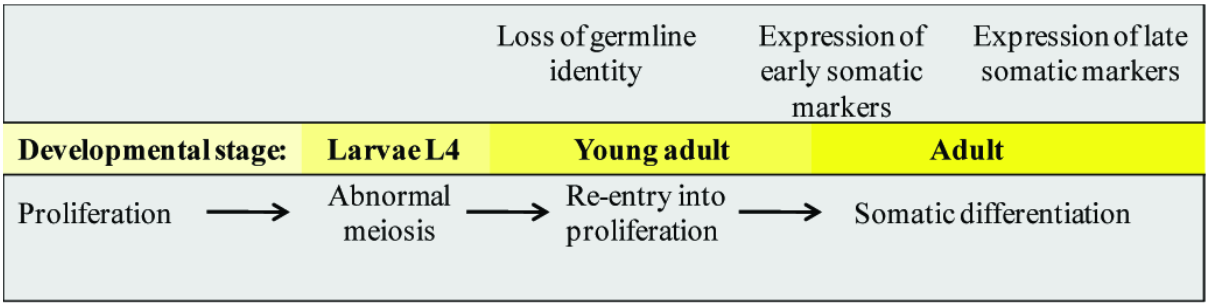
Fig.11 Re-entry into mitosis is required for transdifferentiation.



Gonads were defined into two categories by the appearance of the nuclei in the DAPI staining. Gonads that contain cells with small nuclei (~4µm) were defined as proliferating (=A), and gonads that only contained cells with increased nuclei size (>5.0µm) were defined as arrested (=B). The graph shows the percentage of gonads which were positively or negatively stained for HLH-1 at TP2.25.

In summary this analysis shows that MEX-3 has an underlying function in promoting mitosis and in maintaining germline identity; a function which becomes obvious in germ cells lacking the major regulators (GLP-1, GLD-1, and GLD-2) of the mitosis/meiosis decision. Furthermore this study suggests that germ cells undergoing germ line to soma transition, and hence teratoma formation, go through different consecutive cell cycle stages. After an initial phase of proliferation, germ cells enter meiosis, however they fail to progress through meiosis, re-enter mitosis, and undergo germ line to soma transition (Fig.12, next page)

Fig.12) Germ line to soma transition is an orderly, multistep process.



b. Translational repression of Cyclin E prevents precocious mitosis and embryonic gene activation during *C.elegans* meiosis.

i. Introduction to the published manuscript.

The *gld-1 (q485)* mutant is the simplest genetic background in which, at low frequency, germ line to soma transition can be observed (Ciosk et al. 2006). Therefore we asked whether also in the *gld*-1 mutant germ line to soma transition occurs in the sequential order as described before, whether there is a relationship between the cell cycle and the loss of germ cell identity, and whether GLD-1 directly regulates cell cycle factors.

For the purpose of our analysis we used a feminized *gld-1* mutant background, to ensure that transdifferentiation does not result from abnormal fertilization. Our analysis of the *gld-1, fem-1* mutants showed that germ cells undergoing germ line to soma transition recapitulate the different cell cycle stages we had described before in the quadruple mutant. Germ cells exit meiosis through an abnormal M-phase, proliferate and undergo teratomatous differentiation. Analysis of core cell cycle factors as potential GLD-1 targets showed that GLD-1 binds to mRNAs encoding *cye-1/Cyclin E*, as well as *cyb-2.1, cyb-2.2, and cyb-3/Cyclin Bs*. Further we found that the lack of translational repression of CYE-1 through GLD-1 leads to premature activation of CDK-2 which promotes the meiosis to mitosis transition, and which is required for teratoma formation. Interestingly we found that precocious embryonic gene activation, an early marker for germ line to soma transition, occurs already with the re-entry into mitosis and is independent of proliferation.

Previously muscle transdifferentiation in the *mex-3, gld*-1 mutant had been found to depend on PAL-1/Caudal (Ciosk et al. 2006), while leaky PAL-1 expression in the wild type gonad is not sufficient to induce muscle TD (Mootz et al. 2004). Our result let us propose that the translational regulator GLD-1 maintains germline identity and prevents tumor formation through translational repression of several targets, such as CYE-1 (our study) and PAL-1 (Mootz et al. 2004). Loss of this control leads to re-entry into mitosis and precocious EGA, which creates an environment that allows a somatic determinant like PAL-1 to promote teratomatous differentiation. This suggests that the loss of translational control and the ectopic expression of various targets not directly lead to germ line to soma transition, but first a change in the transcriptional competence of the cells is induced and is required for a cell fate change.

Declaration:

The experiments in this publication were performed by Bjoern Biedermann, with the following exceptions; Mathias Senften performed the GLD-1 immunoprecipitation and the biotin-RNA assay; Jane Wright did the RNA quantification of the GLD-1 targets, as well as of the embryonic genes by qPCR.

ii. Copy of the published manuscript.

Developmental Cell
Article

Translational Repression of Cyclin E Prevents Precocious Mitosis and Embryonic Gene Activation during *C. elegans* Meiosis

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SUMMARY

Germ cells, the cells that give rise to sperm and egg, maintain the potential to recreate all cell types in a new individual. This wide developmental potential, or totipotency, is manifested in unusual tumors called teratomas, in which germ cells undergo somatic differentiation. Although recent studies have implicated RNA regulation, the mechanism that normally prevents the loss of germ cell identity remains unexplained. In *C. elegans*, a teratoma is induced in the absence of the conserved RNA-binding protein GLD-1. Here, we demonstrate that GLD-1 represses translation of CYE-1/cyclin E during meiotic prophase, which prevents germ cells from re-entering mitosis and inducing embryonic-like transcription. We describe a mechanism that prevents precocious mitosis in germ cells undergoing meiosis, propose that this mechanism maintains germ cell identity by delaying the onset of embryonic gene activation until after fertilization, and provide a paradigm for the possible origin of human teratomas.

INTRODUCTION

The production of eggs and sperm involves a special cell division, meiosis, which produces haploid cells from diploid precursors. Simple eukaryotes undergoing meiosis, such as budding yeast, can revert to mitosis without completing meiotic division as late as after recombination (Esposito, 2006; Simchen, 2009). However, such flexibility could lead to cancer in organisms whose gametes develop within a specialized organ, the gonad, in which the consecutive stages of germ cell development (proliferation, meiosis, and differentiation into the gametes) occupy distinct compartments. In such organisms, surprisingly little is known about how re-entry into mitosis is inhibited once cells have started meiosis. During *Drosophila* oogenesis, the re-entry into mitosis is prevented, at least in part, through translational repression of cyclin A by the RRM-type RNA-binding protein Bruno (Sugimura and Lilly, 2006). During *C. elegans* oogenesis, the re-entry into mitosis is prevented by an unrelated, maxi-KH, RNA-binding protein GLD-1 (Francis et al., 1995), which belongs to the STAR (signal transduction and activation of RNA) protein family that includes mammalian Quaking and Sam68 (Vernet and Artzt, 1997).

Remarkably, some germ cells in the *gld-1* mutant differentiate into various somatic cells, bypassing the normal program of oocyte formation and fertilization (Figure 1) (Ciosk et al., 2006). This phenotype, referred to as the worm teratoma, is enhanced by the loss of another conserved RNA-binding protein, MEX-3 (Ciosk et al., 2006). Because GLD-1 functions as a translational repressor (for example, Lee and Schedl, 2001; Marin and Evans, 2003; Schumacher et al., 2005), abnormal expression of its target mRNA(s) in a GLD-1(−) germ line is predicted to cause re-entry into mitosis and teratomatous differentiation. However, the identity of mRNA(s) whose repression is critical to prevent mitosis, and the relation between the cell cycle and the loss of germ cell identity, remains unclear.

In this study, we show that GLD-1 controls the cell cycle, at least in part, by translational repression of *cye-1*/cyclin E mRNA. In the *gld-1* mutant, derepression of CYE-1 results in ectopic activation of CYE-1/CDK-2, which promotes a precocious entry into the M phase of the cell cycle, consequently inducing a germline tumor. Remarkably, we find that the reactivation of the cell cycle leads to transcriptional activation of genes that are normally expressed only in the early embryo. These findings suggest that preventing mitosis during meiotic prophase is crucial not only for tumor suppression but also for maintenance of germ cell identity.

RESULTS

The *C. elegans* gonad contains proliferating stem cells in the distal-most region and cells at consecutive stages of meiotic prophase more proximally (Figure 1) (Seydoux and Schedl, 2001). The medial region of a wild-type gonad consists largely of germ cells at the pachytene stage of meiosis. In adults, germ cells in the proximal gonad differentiate into oocytes, which remain arrested at diakinesis of meiotic prophase until fertilization. This orderly progression between developmental stages is disrupted in the *gld-1* mutant that harbors a germline tumor (Francis et al., 1995). Proximally, the tumor consists mostly of undifferentiated germ cells. Additionally, variable numbers of ectopic (teratomatous) somatic cells are typically sandwiched

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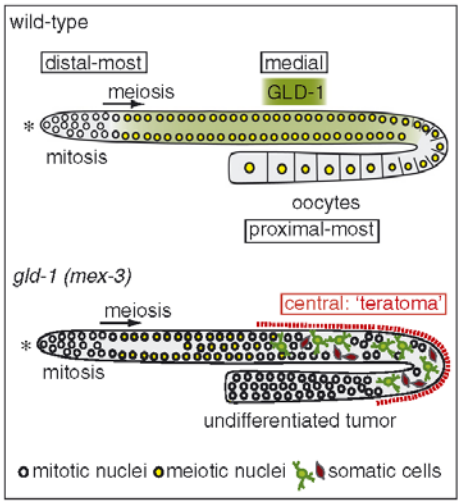

Figure 1. GLD-1 Prevents Re-Entry into Mitosis and Teratomatous Differentiation of Germ Cells

"Distal-most," "medial," and "proximal-most" indicate parts of a wild-type adult gonad. "Central" indicates the region that typically contains ectopic somatic (teratomatous) cells in a *gld-1* or *gld-1 mex-3* gonad. Asterisks here and in the following figures indicate the distal-most gonad.

between the meiotic cells (distally) and the undifferentiated tumor (proximally) (Ciosk et al., 2006). For the purpose of this paper, we refer to this region as the "central gonad" (Figure 1).

Reversal to Mitosis in GLD-1(−) Germ Cells

To understand the origin of teratomatous cells, we examined the central gonads of *gld-1(q485); fem-1(hc17)* animals in a time course experiment. The feminizing *fem-1(hc17)* background was used to eliminate spermatogenesis (Nelson et al., 1978), ensuring that the ectopic somatic cells are due to teratomatous differentiation and not abnormal fertilization. We first monitored the cell cycle status of germ cells. We followed chromosome condensation by both DAPI staining and histone H3 phosphorylation on serine 10 (PH3) (Hsu et al., 2000), and DNA replication by BrdU incorporation. Additionally, we monitored the assembly of microtubule spindles in a *gld-1; fem-1* strain expressing GFP-tagged β-tubulin. We found that in young *gld-1; fem-1* adults, cells in the central gonads were still meiotic; these cells expressed the synaptonemal complex component HIM-3 (Zetka et al., 1999) (see Figure S2B available online) and displayed no PH3 modification, incorporation of BrdU, or spindle formation (Figure 2A; data not shown). In contrast, starting from 0.5–0.75 days after the L4/adult molt, many cells in the central *gld-1; fem-1* gonads contained condensed chromosomes or initiated DNA replication (Figure 2A). In the *gld-1; fem-1* strain expressing GFP-tagged β-tubulin (which somewhat delays re-entry into the cell cycle), we found that many cells with the highly condensed "bar-like" chromosomes also assembled microtubule spindles

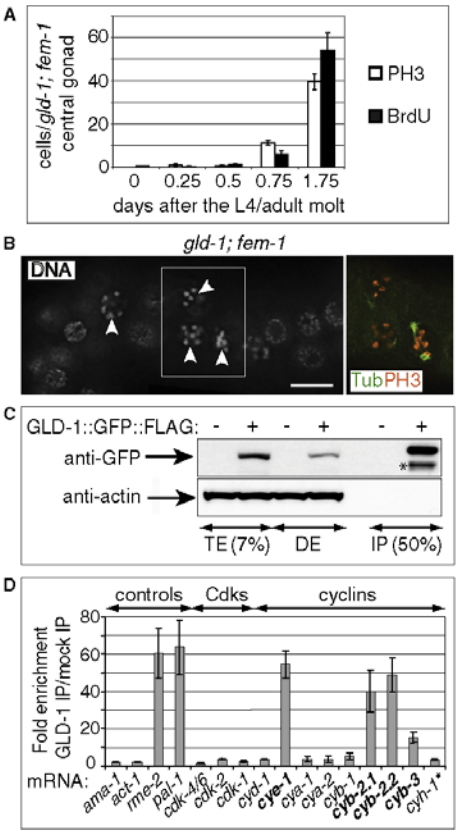

Figure 2. The Role of GLD-1 as a Germline Tumor Suppressor May Involve Translational Regulation of Cyclins E and B

(A) The timing of the meiosis-to-mitosis transition in the central gonad of *gld-1; fem-1* animals. The numbers indicate the mean of nuclei per central gonad that either stained for the histone H3 phosphorylation on serine 10 (PH3), or (in an independent experiment) incorporated BrdU; 7–62 and 10–32 gonads were counted for PH3 and BrdU, respectively. Error bars indicate SEM.

(B) Germ cells in the central *gld-1; fem-1* gonad enter the M phase of the cell cycle. The left panel shows a partial gonad from a 1-day-old adult stained by DAPI (DNA). Arrowheads mark nuclei with highly condensed, bar-like chromosomes. The boxed area, magnified on the right, shows PH3 (red) and GFP-tagged β-tubulin (Tub, green). Scale bar: 10 μm.

(C) GLD-1 immunoprecipitation. Extracts from worms expressing either the endogenous (nontagged) GLD-1, or a rescuing GFP-FLAG-tagged GLD-1, were subjected to immunoprecipitation with FLAG antibodies. The western blot shows GLD-1::GFP::FLAG protein levels in the total extracts (TE), FLAG-depleted extracts (DE), and FLAG-immunoprecipitates (IP). *Degradation product.

(D) GLD-1 associates with mRNAs encoding cyclins E and B. Enrichment of each mRNA in the GLD-1-containing versus GLD-1-missing (mock) immunoprecipitate was determined by quantitative RT-PCR. Each bar represents the mean of three experiments, and the error bars are SEM. *ama-1* and *act-1* are negative controls, and *rme-2* and *pal-1* are known GLD-1 targets. *CYH-1 role in the cell cycle is unclear.

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(Figure 2B). Two lines of evidence suggest that chromosome condensation preceded DNA replication. First, we noticed that although the number of bar-like chromosomes in *gld-1; fem-1* germ cells was typically greater than in wild-type oocytes (which until fertilization maintain 6 bivalents), we never observed more than 12 individual chromosomes (Figures 2B and 3D). This suggests defective homolog pairing but not reduplication of DNA. Second, at the time of initial re-entry into mitosis (in 0.5-day-old animals), 13/21 *gld-1; fem-1* gonads that contained nuclei with the condensed chromosomes displayed no BrdU incorporation, while 7/8 gonads that did incorporate BrdU also contained nuclei with condensed chromosomes. Thus, *gld-1; fem-1* germ cells in the central gonad appear to exit meiosis through an abnormal nuclear division, hereafter referred to as the 1st M phase, without a prior DNA rereplication.

GLD-1 Associates with mRNAs Encoding Cyclins E and B

Because GLD-1 is known to function as a translational repressor, we asked if GLD-1 regulates the cell cycle by repressing translation of key cell cycle regulators: cyclins and cyclin-dependent kinases. To address this, extracts from either wild-type or transgenic worms that expressed a rescuing FLAG and GFP-tagged GLD-1 (Schumacher et al., 2005) were subjected to immunoprecipitation with anti-FLAG antibodies (Figure 2C). Abundance of mRNAs that coprecipitated with GLD-1 was determined by quantitative RT-PCR. We found that mRNAs encoding cyclin E (*cye-1*) and three B-type cyclins (*cyb-2.1*, *cyb-2.2*, and *cyb-3*) were strongly enriched in the IPs from the transgenic worm extracts, when compared to IPs from the wild-type extracts (Figure 2D). To eliminate any experimental bias from comparing IPs between different strains, extracts from the transgenic worms were also subjected to immunoprecipitation with either FLAG or MYC antibodies (Figure S1A). As above, *cye-1*, *cyb-2.1*, *cyb-2.2*, and *cyb-3* mRNAs were enriched in the anti-FLAG IPs when compared with the anti-MYC IPs (Figure S1B). These results suggest that GLD-1 may repress translation of cyclins E and B.

CYE-1/CDK-2 Promotes the Meiosis-to-Mitosis Transition in *gld-1* Germ Cells

Cyclins E and B regulate cyclin-dependent kinases Cdk2 and Cdk1, respectively (Boxem, 2006). Thus, ectopic translation of these cyclins in the *gld-1* mutant may result in ectopic activation of CDK-2 and CDK-1. To test the relative importance of CDK-2 and CDK-1 for the 1st M phase, we depleted CDK-2 by RNAi, or inactivated CDK-1 by a temperature-sensitive allele (*ne2254*), in the *gld-1; fem-1* animals expressing GFP-tagged β-tubulin. While the central gonad of a 1-day-old *gld-1; fem-1* animal (n = 22) contained on average 5 (±1) PH3-positive nuclei and 3 (±0.5) cells with spindles (Figure 2B), a comparable *gld-1; cdk-2(RNAi); fem-1* gonad (n = 35) contained 1 (±0.4) PH3-positive nuclei and 0.5 (±0.1) cells with spindles (Figure 3A). This suggests that CDK-2 promotes the 1st M phase. To test if this role of CDK-2 requires CYE-1/cyclin E, we examined *gld-1; cye-1(RNAi); fem-1* gonads (n = 30) and found that they contained only 0.1 (±0.1) PH3-positive nuclei and 0.2 (±0.1) cells with spindles (Figure 3B). Thus, similar to CDK-2, CYE-1 promotes re-entry into mitosis. This became even more striking in older *gld-1; cye-1(RNAi); fem-1* gonads, in which the whole central part was filled with large, arrested, meiotic-like cells, with no evidence of mitotic chromosome condensation, spindle formation, DNA replication, or cell death (Figure S3; data not shown). Importantly, *cye-1* RNAi (under conditions used in this study) did not appear to affect the initial proliferation and entry into meiosis in *gld-1; fem-1* gonads (Figures S2A–S2C).

To examine the effect of CDK-1 inactivation, we analyzed the central gonads of 1-day-old *gld-1; fem-1* (n = 18) and *gld-1; cdk-1; fem-1* (n = 21) animals that were shifted to the restrictive temperature for *cdk-1(ne2254)*. We found that inactivation of CDK-1 only moderately affected the 1st M phase, as the gonads contained, respectively, 24 (±2.5) versus 11 (±1.6) PH3-positive

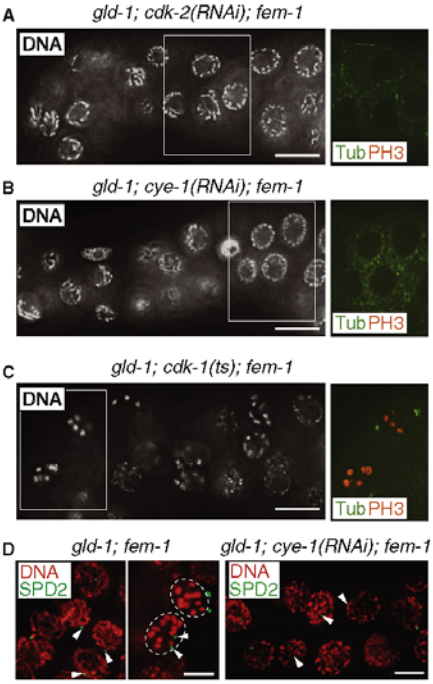

Figure 3. CYE-1 and CDK-2 Drive Re-Entry into Mitosis in *gld-1* Germ Cells

(A–C) CYE-1 and CDK-2, but not CDK-1, are essential for chromosome condensation and spindle assembly in the central *gld-1; fem-1* gonad. Shown are deconvolved fluorescence micrographs obtained from 1-day-old adults. The left panels show partial gonads stained by DAPI (DNA). The boxed areas, magnified on the right, show PH3 (red) and GFP-tagged β-tubulin (Tub, green). Scale bars: 10 μm.

(D) CYE-1 promotes centrosome "duplication" in *gld-1; fem-1* germ cells. Shown are maximum intensity projections of deconvolved fluorescence micrographs. The gonads were stained with DAPI (DNA, red) and for the constitutive centrosome component SPD-2 (green). In the *gld-1; cye-1(RNAi); fem-1* germ cells centrosome duplication is inhibited. Arrowheads point to some SPD-2-stained centrosomes. Each dotted circle demarcates a single nucleus. Scale bars: 5 μm.

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cells and 20 (±2.4) versus 8 (±1.2) cells with spindles (Figure 3C; data not shown). While these results do not exclude a potential role for CDK-1 in the exit from the M phase and/or subsequent proliferation, they suggest that CDK-1 is not critical for entry into the 1st M phase.

In somatic cells, a typical mitotic spindle is organized by centrosomes, whose assembly depends on cyclin E/Cdk2 (Cowan and Hyman, 2006; Tsou and Stearns, 2006). However, during *C. elegans* meiosis, centrosomes are eliminated around diakinesis, so assembly of the meiotic spindle is independent of centrosomes (Albertson and Thomson, 1993). To determine if centrosomes are present during the 1st M phase, we stained the gonads for the constitutive centrosomal component SPD-2 (Kemp et al., 2004). Most (92%; n = 60) *gld-1; fem-1* cells with the condensed bar-like chromosomes contained two centrosomes (Figure 3D). In contrast, *gld-1; cye-1(RNAi); fem-1* cells (96%; n = 383) contained only a single SPD-2-stained centrosome (Figure 3D). This was similar to *gld-1; fem-1* germ cells in which chromosomes have not yet condensed (97%; n = 305) (Figure 3D), or to wild-type pachytene and diplotene germ cells (data not shown). Thus, CYE-1 (and presumably CDK-2) promotes centrosome duplication and/or separation, and mitotic-like spindle assembly during the 1st M phase. Together, our findings suggest that CYE-1/CDK-2 activity drives entry into the 1st M phase in the central gonad.

Repression of CYE-1 in the Central Gonad Requires GLD-1

To test if GLD-1 binding to *cye-1* mRNA prevents CYE-1 expression in the wild-type germ line, we first analyzed gonadal expression of CYE-1. We confirmed a previous report that CYE-1 is expressed in the distal-most and proximal gonad, but is not expressed in the medial gonad that contains GLD-1 (Figure 4A) (Brodigan et al., 2003). We found that this medial repression depends on GLD-1, as CYE-1 was no longer repressed in *gld-1* mutants or *gld-1* RNAi knocked-down animals (Figure S4A; Figure 4A). Importantly, in wild-type gonads CYE-1 is never detected in the leptotene/zygotene and early pachytene nuclei. In contrast, such nuclei were beginning to express CYE-1 in *gld-1(RNAi)* gonads (Figures 4A and 4B). Because the loss of GLD-1 did not cause an increase in *cye-1* mRNA levels (Figure S4B), these results suggest that GLD-1 mediates repression of *cye-1* mRNA translation.

GLD-1 Associates with the 3′UTR of *cye-1* mRNA through GLD-1-Binding Elements

To test if this repression is mediated by GLD-1 binding to *cye-1* mRNA, we tested whether the rescuing FLAG and GFP-tagged GLD-1 (for simplicity GLD-1) copurified with in vitro produced *cye-1* mRNA added to a worm extract. Indeed, we found that GLD-1 copurified with the complete *cye-1* mRNA or its 3′UTR, but not with the equimolar amounts of 5′UTR, the coding sequence, or the antisense 3′UTR (Figure 5A). These results suggest that GLD-1 associates with the *cye-1* mRNA via its 3′UTR. To identify the RNA elements responsible for GLD-1 binding, we divided the 3′UTR into four fragments (F1–F4; Experimental Procedures), and tested their binding to GLD-1. Two fragments, F1 and F3, were found to associate with GLD-1 (Figure 5B). Previous studies showed that recombinant GLD-1

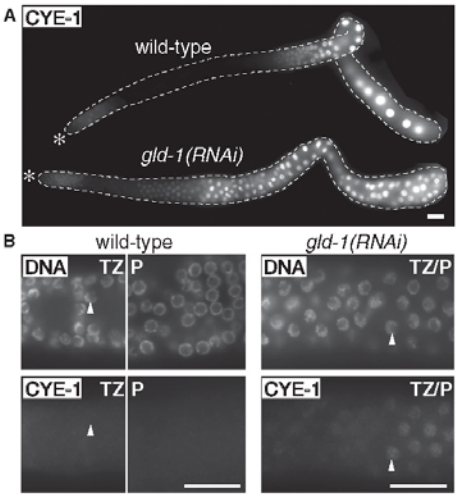

Figure 4. GLD-1 Represses Expression of CYE-1

(A) Micrographs show gonads (outlined), dissected from 1-day-old wild-type adults that were subjected to either mock or *gld-1* RNAi. The gonads were immunostained for CYE-1. Note that in the *gld-1(RNAi)* gonad GLD-1 was knocked down to the level that caused derepression of CYE-1 but not ectopic proliferation. Scale bar: 20 μm.

(B) Magnification of germ cells, from (A), which are in the early (transition zone [TZ]) and later (pachytene [P]) stages of meiotic prophase. Arrowheads point to TZ nuclei (with the crescent-shaped DNA) that either do not (wild-type) or do (*gld-1* RNAi) express CYE-1. Scale bars: 20 μm.

can bind the UACU(C/A)A sequence (Ryder et al., 2004). We noticed that an identical sequence (S3) is present in the F3 fragment, and that fragments F1 and F3 each contain an additional, related sequence (S1 and S2, respectively; Figure 5C; Experimental Procedures). To test if these sequences are involved in GLD-1 binding, we mutated sequence S1 (to S1mt) and deleted both S2 and S3 (ΔS2-3). We found that the ΔS2-3 deletion strongly reduced GLD-1 binding and that residual binding was further reduced by the S1mt mutation (Figure 5C). We conclude that GLD-1 binds the *cye-1* 3′UTR and that this association likely involves the putative GLD-1-binding elements.

GLD-1-Binding Elements in the *cye-1* 3′UTR Confer Repression on a GFP Reporter

To ensure that the *cye-1* 3′UTR is required for translational repression in vivo, we created transgenic strains expressing in the germ line a GFP-tagged reporter, whose coding sequence was fused to different 3′UTRs. We found that the *cye-1* 3′UTR, but not the control *tbb-2* (tubulin) 3′UTR, caused repression in the medial gonad (Figures 5D and 5E). Importantly, this repression was eliminated when the reporter was fused to the mutated *cye-1* 3′UTR (S1mt ΔS2-3) that no longer bound GLD-1 and in *gld-1(RNAi)* animals (Figure 5F; Figure S4C). These results suggest that GLD-1 represses *cye-1* mRNA translation, which presumably prevents CDK-2 activation.

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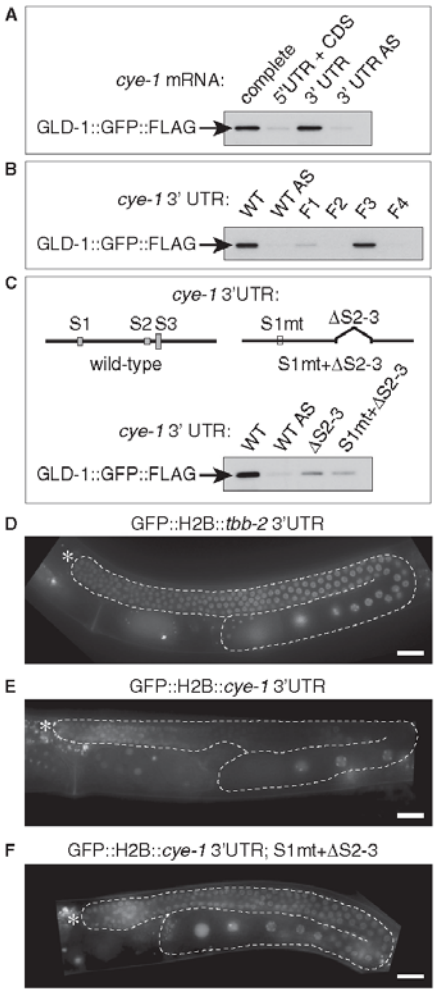

Figure 5. GLD-1 Binding to the 3′UTR of *cye-1* mRNA Results in Its Translational Repression

A–C show western blots of GLD-1::GFP::FLAG coprecipitated with biotinylated RNA. D–F show micrographs of wild-type gonads (outlined) from live transgenic worms expressing a GFP-tagged reporter, whose coding sequence was fused to different 3′UTRs.

(A) GLD-1 associates with the 3′UTR of *cye-1* mRNA. GLD-1 association is mediated by the sense, but not antisense (AS), 3′UTR. "Complete" indicates 5′UTR + coding sequence + 3′UTR.

(B) Two fragments within the *cye-1* 3′UTR, F1 and F3 (Experimental Procedures), are responsible for association with GLD-1.

(C) Putative GLD-1-binding sites (S1 and S2–S3; the higher the rectangle the better similarity to the putative GLD-1 binding sequence) in fragments F1 and F3 are required for GLD-1 binding. Deletion of sites S2–S3 (ΔS2-3) strongly reduced GLD-1 binding, and the residual binding was further reduced by a mutation in S1 (S1mt+ΔS2-3). "WT" indicates the wild-type *cye-1* 3′UTR.

(D) The *tbb-2* (tubulin) 3′UTR fused to the GFP::H2B allowed reporter expression throughout the gonad.

(E) The *cye-1* 3′UTR conferred translational repression on the GFP::H2B reporter in the medial gonad.

(F) The S1mt+ΔS2-3 *cye-1* 3′UTR (which did not bind GLD-1 in vitro) no longer conferred translational repression on the GFP::H2B reporter. Scale bars: 20 μm.

CYE-1 Is Required for Teratoma Formation

Finally, to determine if derepression of CYE-1/CDK-2 activity in the *gld-1* mutant is important for teratoma formation, we stained gonads for muscle myosin and UNC-10 (a component of the postsynaptic vesicles) to detect muscles and neurons, respectively (Koushika et al., 2001; Priess and Thomson, 1987). As expected, we found that most 1.75-day-old *gld-1; fem-1* gonads (84%; n = 19) contained ectopic muscles and/or neurons (Figure 6A). In contrast, very few *gld-1; cye-1(RNAi); fem-1* gonads (5%; n = 22) contained any muscles or neurons (Figure 6A). We also tested expression of HLH-1/MyoD, which in the embryo is expressed early in the muscle-specification pathway (Krause et al., 1990). We found that, similar to the terminal markers, expression of HLH-1 depended on CYE-1 (Figure 6B). While, on average, a 1.75-day-old mock-treated gonad (n = 18) contained 10.4 (±3.2) HLH-1-positive nuclei, a *cye-1* RNAi-treated gonad (n = 15) had 0.3 (±0.3) such nuclei.

Previously, germ cells destined to undergo teratomatous differentiation were shown to lose germ line-specific RNA/protein structures called P granules (Ciosk et al., 2006). To test if CYE-1 is required for the loss of P granules, we examined the central gonads for expression of the constitutive P granule component PGL-1 (Kawasaki et al., 1998). Consistent with previous results, we found that PGL-1 was diminished in most cells in the central *gld-1; fem-1* gonad. In contrast, most if not all *gld-1; cye-1(RNAi); fem-1* cells continued to express PGL-1, which (similar to wild-type P granules) was enriched in perinuclear speckles (Figure 6C). At least one additional germ line-expressed protein, the yolk receptor RME-2, was expressed in the *gld-1; cye-1(RNAi); fem-1* cells, but was diminished in *gld-1; fem-1* cells (data not shown). These results suggest that CYE-1/CDK-2 activity is required for the loss of germ cell characteristics and teratoma formation.

Re-Entry into Mitosis Results in a Precocious Onset of Embryonic Gene Activation

Acquisition of a non-germ line identity in the early embryo is manifested by the onset of embryonic gene activation (EGA). In worms, EGA is characterized by transcription of several very early transcripts, such as *vet-1*, *vet-4*, and *vet-6* (Seydoux et al., 1996). To test if the loss of germ cell identity is accompanied by a precocious onset of EGA, we compared, by quantitative RT-PCR, the levels of *vet-1*, *vet-4*, and *vet-6* mRNAs in gonads dissected from 0.5-day-old or 1.5-day-old, *gld-1; fem-1* and *gld-1; cye-1(RNAi); fem-1* animals. We found that the *gld-1; fem-1* gonads from 1.5-day-old animals had much higher levels of *vet-1*, *-4*, and *-6*, than the comparable *gld-1; cye-1(RNAi); fem-1* gonads (Figure 7A). This enrichment correlated with re-entry into mitosis, as the gonads from 0.5-day-old *gld-1; fem-1* and *gld-1; cye-1(RNAi); fem-1* animals contained similar

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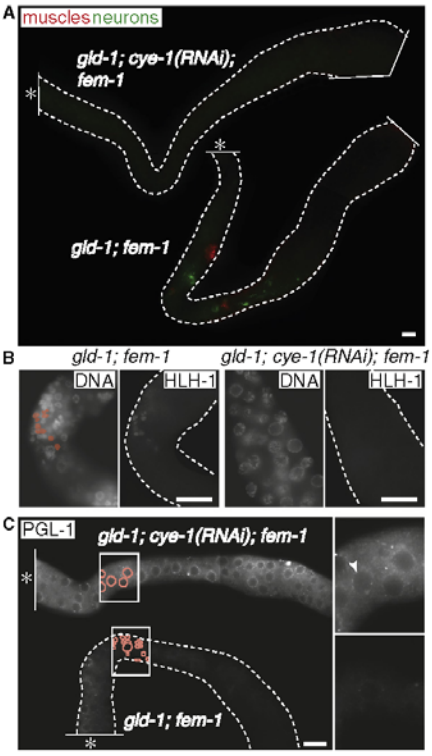

Figure 6. CYE-1 Promotes the Loss of Germ Cell Identity and Teratoma Formation in *gld-1* Gonads

(A) Differentiation into muscles and neurons in a teratoma requires CYE-1. Shown are maximum intensity projections of *gld-1; fem-1* gonads (outlined) from 1.75-day-old animals subjected to mock (lower) or *cye-1* (upper) RNAi. The gonads were stained with antibodies to visualize muscle myosin (red) and neuronal postsynaptic vesicles (green). Solid lines (also in C) indicate where the images were cropped to facilitate the alignment. Scale bar: 15 μm.

(B) Early steps of muscle differentiation in a teratoma require CYE-1. Shown are partial *gld-1; fem-1* gonads (outlined) from 1.75-day-old animals subjected to mock or *cye-1* RNAi. The gonads were stained with DAPI (DNA) and antibody against HLH-1/MyoD. Notice that HLH-1 was expressed in small, post-meiotic *gld-1; fem-1* nuclei; these nuclei were false-colored on the left panel. Scale bars: 20 μm.

(C) CYE-1 promotes the loss of germ line-specific RNP (P) granules in the central *gld-1; fem-1* gonad. Shown are 1.5-day-old central gonads, subjected to mock (lower) or *cye-1* (upper) RNAi, that were stained with antibody to visualize PGL-1, a constitutive component of P granules. Notice that PGL-1 was diminished in most cells in the central *gld-1; fem-1* gonad, but continued to be present in the same region in the *gld-1; cye-1(RNAi); fem-1* gonad. The circular drawings within the boxed areas indicate nuclei. The boxed areas are magnified on the right. Arrowhead points to a perinuclear P granule. Scale bar: 20 μm.

low levels of *vet-1*, *-4*, and *-6* mRNA (Figure 7A). These results suggest that *gld-1* germ cells express at least some mRNAs normally transcribed in the early embryo, and that their transcription depends on CYE-1 activity.

If the onset of embryonic-like transcription is related to teratoma formation, the embryonic transcripts are expected to be present in the central gonad. To test this, we performed in situ hybridization to detect *vet-4* mRNA. We detected robust expression of *vet-4* mRNA in 1.5-day-old (100%; n = 48), but not 0.5-day-old (18%; n = 28), *gld-1; fem-1* gonads (Figure 7B; data not shown). Remarkably, *vet-4* was expressed mostly in the central gonad, where CYE-1 drives re-entry into mitosis and teratoma formation. In agreement with the quantitative RT-PCR data, we found that the *vet-4* signal was either absent or strongly reduced in the 1.5-day-old *gld-1; cye-1(RNAi); fem-1* gonads (only 12.5% of the gonads had *vet-4* expression and in very few cells; n = 64) (Figure 7B). These results suggest that the abnormal EGA is due either to the CYE-1-dependent re-entry into mitosis, or subsequent proliferation in the tumor.

To distinguish between these two possibilities, *gld-1; fem-1* animals were subjected to RNAi-mediated depletion of a DNA replication-licensing factor, CDT-1 (Zhong et al., 2003). This depletion (under conditions used here) had no obvious effect on the initial proliferation of germline stem cells, or entry into meiosis, in *gld-1; fem-1* gonads (Figure S5). Consistent with the finding that the 1st M phase occurs without prior DNA rereplication (this study), CDT-1 depletion did not prevent re-entry into mitosis, as the central *gld-1; cdt-1(RNAi); fem-1* gonads contained many PH3-positive cells (Figure 7C). However, consistent with a role of CDT-1 in DNA replication, these gonads displayed no BrdU incorporation (Figure 7C) and, consequently, contained similar numbers of cells as the *gld-1; cye-1(RNAi); fem-1* gonads (Figure 7C). Importantly, by quantitative RT-PCR, the CDT-1-depleted *gld-1; fem-1* gonads (in which cells arrested after re-entry into mitosis) contained much higher levels of *vet-1*, *-4*, and *-6* mRNAs than the CYE-1-depleted *gld-1; fem-1* gonads (in which cells arrested before re-entry into mitosis) (Figure 7D). Consistently, 93% (n = 123) of the CDT-1 depleted *gld-1; fem-1* gonads showed robust expression of *vet-4*, in contrast to only 21% (n = 112) of CYE-1-depleted *gld-1; fem-1* gonads that showed much weaker *vet-4* expression (Figure 7E). Together, these results suggest that the switch in germ cell identity, as detected by the abnormal EGA, is driven by re-entry into mitosis.

A Model for Teratoma Formation

Our results suggest that derepression of CYE-1/CDK-2 activity during progression through meiosis results in re-entry into mitosis and a precocious onset of EGA. Previously, differentiation into muscles in a worm teratoma was shown to depend on the maternal transcription factor PAL-1/Caudal (Ciosk et al., 2006), whose translation in the wild-type germ line is repressed by GLD-1 (Mootz et al., 2004). However, PAL-1 is occasionally expressed in a wild-type germ line but is apparently insufficient to induce muscle differentiation (Mootz et al., 2004). Thus, we propose that the acquisition of embryonic-like identity, manifested by the onset of EGA, is required for somatic fate determinants, including but not limited to PAL-1, to induce teratomatous differentiation into various somatic cell types (Figure 7F).

DISCUSSION

Cyclin E/Cdk2 and the Meiosis/Mitosis Decision

Our findings demonstrate that a tight regulation of CDK-2 activity is critical for normal progression through meiosis. This regulation

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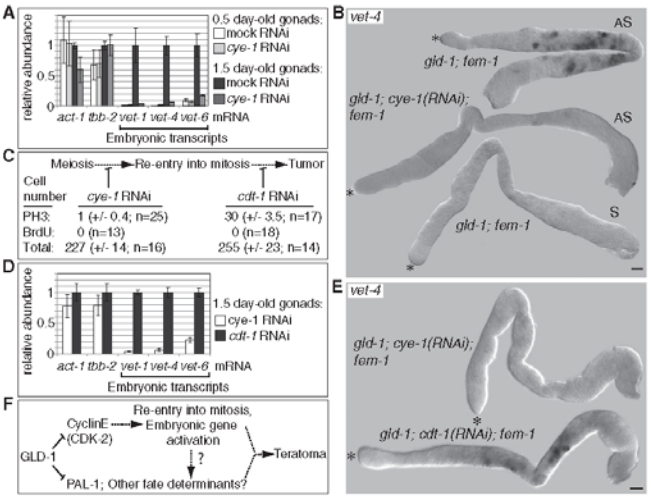

Figure 7. The Re-Entry into Mitosis Promotes a Precocious Onset of Embryonic Gene Activation

(A) Three mRNAs, *vet-1*, *vet-4*, and *vet-6*, which are normally expressed in the early embryo, are expressed in *gld-1; fem-1* but not *gld-1; cye-1(RNAi); fem-1* gonads. The graph shows relative amounts of mRNAs, determined by quantitative RT-PCR, found in 0.5- or 1.5-day-old *gld-1; fem-1* gonads subjected to either mock or *cye-1* RNAi. The levels were normalized to the values obtained from the 1.5-day-old *gld-1; fem-1*, mock RNAi gonads. Each bar represents the mean of three independent biological replicates, and the error bars represent SEM.

(B) *vet-4* mRNA is expressed in the central *gld-1; fem-1* gonad and this expression depends on CYE-1. Shown are light micrographs of *gld-1; fem-1* gonads from 1.5-day-old animals subjected to either mock or *cye-1* RNAi. The gonads were hybridized with antisense (AS) or sense (S) probes for the *vet-4* mRNA. Scale bar: 20 μm.

(C) Depletion of CDT-1 arrests tumor formation in the central gonad after the 1st M phase. The values correspond to the mean number of cells (±SEM) that, in the central gonads of CYE-1- or CDT-1-depleted 1.5-day-old *gld-1; fem-1* animals, displayed PH3 modification or incorporated BrdU. "Total" cell numbers were determined as explained in Experimental Procedures.

(D) *vet-1*, *vet-4*, and *vet-6* are expressed in *gld-1; cdt-1(RNAi); fem-1* but not *gld-1; cye-1(RNAi); fem-1* gonads. The graph shows relative amounts of indicated mRNAs in 1.5-day-old *gld-1; fem-1* gonads subjected to either *cdt-1* or *cye-1* RNAi. Levels were normalized to the *cdt-1* RNAi values. Each bar represents the mean of four independent biological replicates, and error bars show SEM.

(E) The *vet-4* mRNA is expressed in the central *gld-1; cdt-1(RNAi); fem-1* gonad. Shown are light micrographs of *gld-1; fem-1* gonads from 1.5-day-old animals subjected to either *cye-1* or *cdt-1* RNAi. The gonads were hybridized with an antisense probe to the *vet-4* mRNA. Scale bar: 20 μm.

(F) A model for GLD-1 in maintaining germ cell identity. Solid lines represent events in the wild-type germ line, dotted lines events in the *gld-1* mutant. During meiotic prophase, GLD-1 represses expression of CYE-1 and the somatic cell fate determinant PAL-1/Caudal. The loss of GLD-1 results in ectopic activation of CYE-1/CDK-2 that, through unknown targets, promotes the entry into mitosis and premature onset of embryonic-like transcription. This may allow a maternally provided fate determinant like PAL-1, and potentially additional determinants expressed during the abnormal EGA, to direct differentiation into various somatic cell types.

is achieved, at least in part, through the repression of cyclin E translation. This finding is superficially reminiscent of the mechanism reported in flies, where repression of a G2/M phase cyclin (cyclin A) by Bruno has been implicated in preventing germline tumor formation (Sugimura and Lilly, 2006). However, GLD-1 is unrelated to Bruno, and, while GLD-1 binds to and might regulate expression of mRNAs encoding B-type cyclins (this study), it is CDK-2, rather than CDK-1, activity that appears to be primarily responsible for driving mitosis in *gld-1* germ cells destined to form a teratoma. Thus, although translational repression of cyclins appears to be a conserved feature of the meiotic prophase, there appear to be important species-specific differences. It is possible that the regulation of cyclin E in flies is different because, unlike in worms and mammals, the fly germ line contains nurse cells whose endoreplication requires cyclin E expression (Lilly and Duronio, 2005).

An unresolved issue is precisely how ectopic activation of CYE-1/CDK-2 triggers the 1st M phase. Because CYE-1/CDK-2 can facilitate assembly of centrosomes (Cowan and Hyman, 2006), and centrosomes have been implicated in advancing the timing of mitotic entry, one possibility is that a recruitment and/or stabilization of centrosomal proteins might induce the M phase, for example through the recruitment of the Aurora A kinase AIR-1 (Hachet et al., 2007; Portier et al., 2007). According to this model, the regulation of centrosome biology may play a major role in the mitosis/meiosis decision, which has also been hypothesized in yeast (Simchen, 2009).

The Relation between the Cell Cycle and Embryonic Gene Activation

Unexpectedly, CYE-1-dependent re-entry into mitosis is accompanied by embryonic-like transcription. Because depletion of CDT-1 prevents proliferation but not accumulation of embryonic transcripts, EGA appears to be triggered by re-entry into mitosis, rather than subsequent proliferation in the tumor.

How does reactivation of mitosis result in a precocious EGA? Because cyclin E/Cdk2-mediated phosphorylation is known to regulate, among other targets, transcription factors (Hwang and Clurman, 2005), it is possible that CYE-1/CDK-2 might drive embryonic transcription directly by activating a transcription factor involved in EGA. Alternatively, EGA may be induced by a cell cycle event that, at least in the mutant germ line, depends on a prior activation of CYE-1/CDK-2. While different mechanisms have been implicated in controlling EGA in different models, including repression of basic transcription factors (Guven-Ozkan et al., 2008), degradation of maternal mRNAs (Tadros et al., 2007), and induction of gene expression by a sequence-specific transcription factor (Liang et al., 2008), the precise mechanisms are

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far from being well understood. Interestingly, activation of at least some embryonic genes in the murine embryo is thought to depend on the maternally provided cyclin A2 (Hara et al., 2005). Thus, the connection between the cell cycle and a precocious EGA in the mutant germ line may reflect the normal role of the cell cycle machinery in inducing EGA in the wild-type embryo.

Cell Cycle (De)regulation and Teratoma Formation

Our findings suggest that GLD-1-mediated inhibition of re-entry into mitosis is critical to prevent teratomatous differentiation in the worm germ line. Whether a GLD-1-related protein, or another RNA-binding protein, plays a role similar to GLD-1 in the mammalian germ line is not known. In a mouse model for testicular teratoma, the 129 family of inbred strains, loss of the RNA-binding protein DND1 dramatically increases teratoma incidence (Youngren et al., 2005). Interestingly, DND1 has been demonstrated to stabilize and promote expression of the $p27^{Kip1}$ mRNA that encodes a CDK-2 kinase inhibitor (Kedde et al., 2007). Thus, DND1(−) germ cells are expected to have low levels of $p27^{Kip1}$ and higher CDK-2 activity. Therefore, it is tempting to speculate that derepression of CDK-2 activity may also induce proliferation, EGA, and consequently teratomatous differentiation in the mammalian germ line.

EXPERIMENTAL PROCEDURES

Nematode Culture

Standard procedures were used to maintain the N2 strain of *C. elegans*. Typically, worms grown at 20°C were bleached to collect eggs, the larvae were synchronized by starvation (on plates), and allowed to feed at 25°C, unless indicated otherwise. In RNAi experiments, bacterial strains from Open Biosystem (*cye-1*, *cdt-1*) and Ahringer (*cdk-2*) libraries were used. The *gld-1* dsRNA-expressing vector was generated in this study and contains the sequence corresponding to aa 131–305 of GLD-1 (WormBase release 156). In all RNAi experiments, mock-treated animals were fed bacteria harboring empty vector (derived from p129.36). For *cye-1* or *cdk-2* RNAi, larvae were transferred to RNAi feeding plates as L2; we used RNAi rather than mutations because CYE-1 is required for germ line proliferation (Fay and Han, 2000). For *cdt-1* RNAi, larvae were transferred to RNAi feeding plates directly after synchronization. For *gld-1* RNAi, larvae were transferred to RNAi feeding plates as L4s, and worms were cultured at all stages at 20°C. To allow the initial germ line proliferation and entry into meiosis in the *gld-1(q485); cdk-1(ne2254ts)* strain, worms were initially cultured at 20°C and shifted to 25°C as L4s. In all experiments, gonads were examined at indicated ages after the L4/adult molt. Because in the *fem-1(hc17ts)* background the loss of one copy of the *gld-1* gene results in germ line feminization, all strains containing *gld-1* and *fem-1* mutations were propagated by mating with isogenic males.

Mutant Strains

The following strains were described previously: *gld-1(q485)/hT2[qIs48]*, *gld-1(q485)/hT2[qIs48]; fem-1(hc17ts)* (Ciosk et al., 2006), and *gld-1(q485); ozIs2 [gld-1::gfp::flag]* (Schumacher et al., 2005). The *gld-1(q485)/hT2[qIs48]; fem-1(hc17ts); ojIs1[tbb-2::GFP]* strain generated in this study was derived from the strains *gld-1(q485)/hT2[qIs48]; fem-1(hc17ts)* and *unc-119(ed3) ruIs32 [pie-1::GFP::his-11] III; ojIs1[tbb-2::GFP]* (CGC TY3558). The *gld-1(q485)/hT2[qIs48]; fem-1(hc17ts); ojIs1[tbb-2::GFP]* and *cdk-1(ne2257ts)* (Shirayama et al., 2006) strains were used to generate the *gld-1(q485); cdk-1(ne2254ts)/hT2[qIs48]; fem-1(hc17ts); ojIs1[tbb-2::GFP]* strain. We noticed that the fraction of condensed chromosomes in *gld-1; fem-1* gonads is lower in the strain additionally carrying *ojIs1*, which suggests that either the GFP tag and/or the higher β-tubulin levels delay the cell cycle.

Transgenic Constructs and Strains

For the 3′UTR reporter studies, most sequences were generously provided by Geraldine Seydoux (Merritt et al., 2008). The *cye-1* 3′UTR used in this study was determined by 3′RACE and sequencing. To analyze the effect of *cye-1* 3′UTR on GFP::H2B expression, we cloned the *cye-1* 3′UTR (517 bp; Genebank U97194, 9439–9955 bp) plus an additional 80 bp of the downstream genomic sequence. Standard techniques were applied to mutate GLD-1 consensus binding sites at positions 46–51 (S1mt) and to delete nucleotides 283–348 (ΔS2-3). Microparticle bombardment was used to produce transgenic worms. We obtained the following numbers of stable transgenic lines (lines in which all worms expressed GFP in the germ line): *pie-1::GFP::H2B::tbb-2* (1), *pie-1::GFP::H2B::cye-1* (6), *pie-1::GFP::H2B::cye-1 S1mt+ΔS2-3* (1). In addition, we generated several transiently expressing lines (in which either some but not all worms had germline expression or germline expression became silenced over time): *pie-1::GFP::H2B::cye-1* (4), *pie-1::GFP::H2B::cye-1 S1mt+ΔS2-3* (8). Both stable and transiently expressing *pie-1::GFP::H2B::cye-1* lines expressed GFP only in the distal and proximal, but not medial, germ line. Among the transiently expressing *pie-1::GFP::H2B::cye-1 S1mt+ΔS2-3* lines, 4/8 showed GFP expression throughout the germ line, similar to the stable line, while the remaining lines had either lower or no GFP expression in the distal-most region. Although we cannot explain this expression pattern at this time, once expressed, the GFP intensity gradually increases through the medial gonad, thus showing no evidence of repression.

GLD-1 Immunoprecipitation, Western Blotting, and Coprecipitated RNA Isolation

gld-1(q485); ozIs2 [gld-1::gfp::flag] or wild-type (N2) worms were synchronized and harvested as young adults. Antibodies used for immunoprecipitation—mouse anti-GFP (Roche), mouse anti-FLAG M2 (Sigma), or mouse anti-Myc (9E10)—were prebound to protein A sepharose CL-4B (GE Healthcare Bio-Sciences) in extraction buffer (50 mM HEPES [pH 7.4], 100 mM KOAc, 5 mM MgAc, 0.1% Triton X-100, 10% Glycerol [w/v], 20 mM β-glycerophosphate, 3 mg ml^{-1} complete EDTA-free protease inhibitor cocktail [Roche]). For protein extraction, the buffer was supplemented with 2 mM DTT, Pepstatin A, 1 mM Phenylmethyl sulfonylfluoride, 200 units ml^{-1} RNAsin (Promega), and the concentration of the protease inhibitor cocktail was increased to 7 mg ml^{-1}. Worms were homogenized with Dounce homogenizer. 700 μg of a precleared extract (input) was subjected to immunoprecipitation. The immunoprecipitates were washed three times with extraction buffer.

For each western blot, total protein (corresponding to approximately 7% of input) was loaded into the "TE" lane, and the equivalent volume (after antibody-depletion) into the "DE" lane. To obtain IP samples, beads were heated in sample buffer, and one-half of IP (corresponding to approximately 50% of input) was loaded into "IP" lane. For protein detection, we used mouse anti-GFP (Roche), mouse anti-actin (Chemicon), and the horseradish peroxidase conjugated sheep anti-mouse (GE Healthcare) antibodies.

RNA was extracted from beads using TRIzol (Invitrogen). Precipitation efficiency was enhanced by addition of 10 μg total RNA from mouse brain (Stratagene) to each sample.

RT-PCR Quantification of Coimmunoprecipitated mRNAs

cDNAs were generated using random hexamers with the Superscript III reverse transcriptase system (Invitrogen) from 1/4th of each IP and 2.5 μg of each input. Real-time PCR was performed in duplicate upon 1/20th of obtained IP cDNA using Absolute QPCR SYBR green ROX mix (AbGene) on an ABI PRISM 7700 system (Applied Biosystems). PCR reactions were performed with an initial activation step of 15 min at 95°C, then 40 cycles of 20 s at 95°C and 60 s at 60°C. Standard curves for quantification were generated from a serial dilution of input cDNA for each primer pair. The amount of target present in each IP was derived from the standard curve; an average was calculated for the duplicates and then corrected by the corresponding input value. The fold enrichment in each GLD-1 IP was calculated relative to the control IP. At least one primer in each pair (except *cya-2*) is specific for an exon-exon junction. Primer sequences are available upon request.

Quantification of Embryonic mRNAs in Dissected Gonads

RNA was extracted from 50 gonads per strain using the Picopure RNA Isolation Kit (Arcturus). Two independent RT reactions were performed with oligo dT and the ImProm II Reverse transcription system (Promega) using 40 ng of isolated RNA. 1/12th of the subsequent cDNA was used for a quantitative PCR, performed as described in the previous section. Standard curves for

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every primer pair were generated using a serial dilution of cDNA from *unc-30* embryos and were used to determine the amount of each transcript in the gonads. All duplicate values were first averaged, and then values for a particular transcript were normalized by the mean of either the mock RNAi or *cdt-1* RNAi values from 1.5-day-old animals. Error bars show the SEM for at least three biological replicates. Primer sequences are available upon request.

Biotin-RNA Pull-Down

The assay was performed essentially as described (Lee and Schedl, 2001), with the following change: biotinylated RNA probes were generated from PCR-amplified and gel-purified DNA templates containing the T7 consensus sequence in one DNA strand. 400 ng of biotinylated *cye-1* RNA was added to the worm extract. Shorter *cye-1* fragments and deletion mutants were added at equimolar amounts. The *cye-1* 3′UTR fragments F1–F4 correspond to the following sequences: F1, nucleotides 1–166; F2, 117–283; F3, 235–399; and F4, 350–514, where 1 is the first nucleotide after the stop codon. Putative GLD-1-binding elements in the *cye-1* 3′UTR are as follows: in the F1 fragment, S1 (UACUUA); in the F3 fragment, S2 (CAAUUA or AACUUA) and S3 (UACUCA). In the S1mt mutation, the sequence UACUUA was mutated to CGUCUC.

Immunolocalization

Primary antibodies are referenced in the main text. Secondary antibodies were goat anti-mouse alexa-568, anti-rabbit alexa-568, and anti-rabbit alexa-488 (Molecular Probes). In most experiments, gonads were prepared essentially as described previously (Ciosk et al., 2004). For HIM-3 staining, gonads were dissected in phosphate-buffered saline (PBS), frozen on dry ice, fixed first in 100% methanol (−20°C) for 5 min, and then in 3.7% paraformaldehyde, PBS, 0.08 M HEPES, 1.6 mM $MgSO_4$, 0.8 mM EGTA (pH 6.9) at room temperature (RT) for 5 min. Fluorescence and DIC images were captured with a Zeiss ImagerZ1 microscope equipped with an AxiocamMRm (Zeiss). Images in Figures 2B and 3A–3D were acquired and deconvolved with the Delta Vision Core System (Applied Precision). Imaris 6.1.3 (Bitplane) was used to create the maximum intensity projection in Figures 3D and 6A and Figures S2A and S4A. Unless indicated otherwise, images were acquired with the same exposure and processed in Adobe Photoshop CS2 in an identical manner. Because expression of the transgenic β-tubulin::GFP and GFP::H2B is somewhat variable, the images in Figures 2B, 3A–3C, and 5D–5F were individually adjusted to a similar intensity. As SPD-2 staining in the *gld-1; fem-1* nuclei with the condensed chromosomes was much stronger than in the other nuclei, the SPD-2 signal in the middle panel of Figure 3D was adjusted separately from the other two panels.

To determine total numbers of cells in the central *gld-1; cye-1(RNAi); fem-1* and *gld-1; cdt-1(RNAi); fem-1* gonads, DAPI-stained nuclei were counted within a 80 μm-long fragment of a central gonad, and the value was multiplied by the total length of the central gonad/80 μm.

BrdU Incorporation and Labeling

BrdU (Sigma, 50 mM in water) was mixed 2:1 with M9 buffer. Worms were soaked in the BrdU solution for 1 hr at RT, protected from light. Gonads were dissected, frozen on dry ice, incubated in methanol (−20°C) for 1 hr, and washed in PBS with 0.1% Triton X-100 for 15 min. DNA was denatured by incubation in HCl (2 M) for 45 min at RT, and neutralized in 0.1 M borax (Merck) for 10 min. The staining procedure, using anti-BrdU antibody (Sigma), was performed as described above.

RNA In Situ Hybridization

The *vet-4* RNA hybridizations were performed as described by Broitman-Maduro and Maduro (http://www.faculty.ucr.edu/~mmaduro/resources.htm). The probes corresponded to the nucleotides 385–929 of *vet-4*. For *cye-1* RNA hybridization, dissected gonads were fixed in 0.25% glutaraldehyde, 3% formaldehyde, 100 mM K_2HPO_4 (pH 7.2) and hybridized as described (Jones et al., 1996; Lee and Schedl, 2001). The probes corresponded to the nucleotides 151–550 of *cye-1*. A Zeiss AxioImager Z1 microscope equipped with AxioncamMRm REV 2 CCD camera was used to capture images. All images were processed with Adobe Photoshop 7.0 or CS2 in an identical manner.

SUPPLEMENTAL DATA

Supplemental Data include five figures and can be found with this article online at http://www.cell.com/developmental-cell/supplemental/S1534-5807(09)00340-2.

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Developmental Cell *17*

Supplemental Data

Translational Repression of Cyclin E Prevents Precocious Mitosis and Embryonic Gene Activation during *C. elegans* Meiosis

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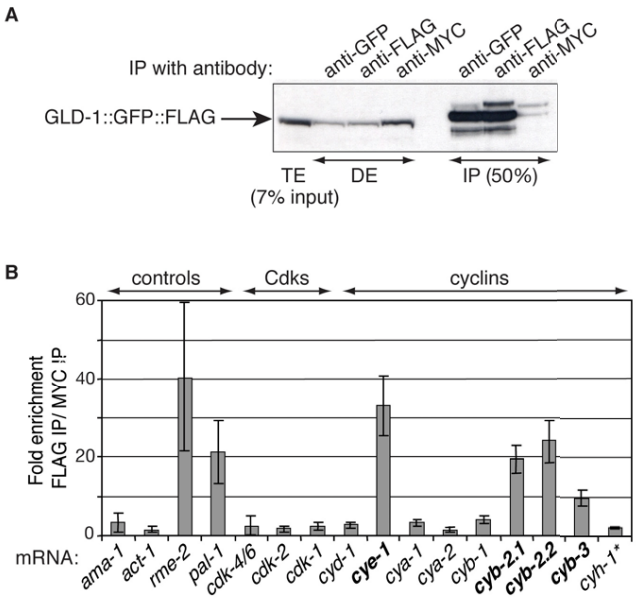


Figure S1.
GLD-1 associates with mRNAs encoding cyclin E and three B-type cyclins. (A) GLD-1::GFP::FLAG is specifically precipitated by anti-GFP and anti-FLAG antibodies, but not by an anti-MYC antibody. Extracts from worms expressing a GFP-FLAG-tagged GLD-1 were subjected to immunoprecipitation with anti-GFP, anti-FLAG, or anti-MYC antibodies. The western blot of total extracts (TE), depleted extracts (DE), and immunoprecipitates (IP) was probed with anti-GFP antibody. (B) GLD-1 associates with mRNAs encoding cyclins E and B. Enrichment of each candidate mRNA in the anti-FLAG versus anti-MYC IP was determined by quantitative RT-PCR. Each bar represents the mean for three independent IPs, and error bars correspond to the standard error of the mean (s.e.m.). *CYH-1/cyclin H role in the cell cycle is not clear. Notice that the messages encoding cyclins E and B associate with GLD-1 similarly to Figure 2D.

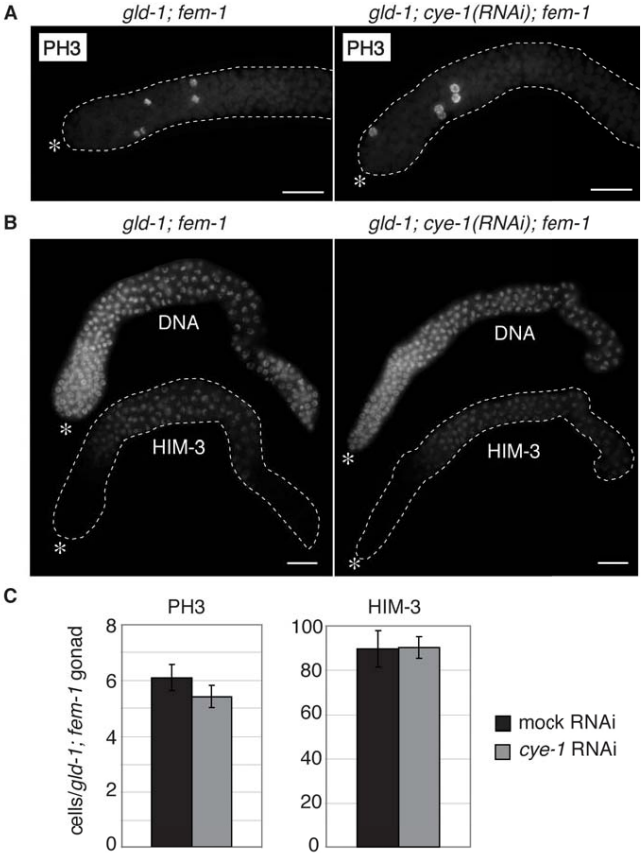


Figure S2. Depletion of CYE-1 by RNAi has little effect on the initial germ cell proliferation in the stem cell compartment or entry into meiosis in *gld-1; fem-1* gonads

(A) The stem cell regions of 0.25 day-old *gld-1; fem-1* gonads contain similar numbers of mitotic cells in *cye-1* RNAi and mock treated animals. Shown are maximum intensity projections of distal gonads (outlined). The gonads were stained with antibody to show PH3 modification (strong signal in the few distal-most nuclei) and DAPI (dark circles in the background). Asterisks here and in the following figures indicate the distal-most gonad. Scale bars: 20 μm.

(B) The *cye-1* and mock RNAi treated, 0.25 day-old, *gld-1; fem-1* gonads have similar numbers of meiotic cells. Shown are fluorescence micrographs of DAPI and HIM-3-stained gonads (outlined). Scale bars: 20 μm.

(C) Quantification of data shown in A and B. Each bar indicates the mean of cells per gonad and the error bars represent the s.e.m. The numbers of scored gonads were, from left to right: 50 and 54 (PH3); 37 and 45 (HIM-3).

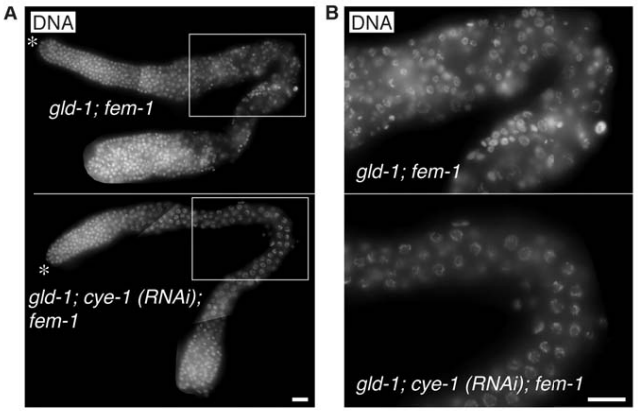


Figure S3. CYE-1 promotes tumor formation in the central *gld-1; fem-1* germ line
(A) Shown are fluorescence micrographs of DAPI-stained *gld-1; fem-1* and *gld-1; cye-1(RNAi); fem-1* gonads from 1.5 day-old animals. Notice that CYE-1 is required for tumor formation in the central gonad. The proximal-most gonad contains undifferentiated germ cells whose proliferation is less dependent on CYE-1; the etiology of the proximal tumor goes beyond the scope of this paper.
(B) Higher magnification of the areas boxed in A. Notice that the *gld-1; fem-1* gonad contains mostly small nuclei that are derived from meiotic cells that have re-entered mitosis. In contrast, the central *gld-1; cye-1(RNAi); fem-1* gonad contains mostly large arrested nuclei. Scale bars: 20 μm.

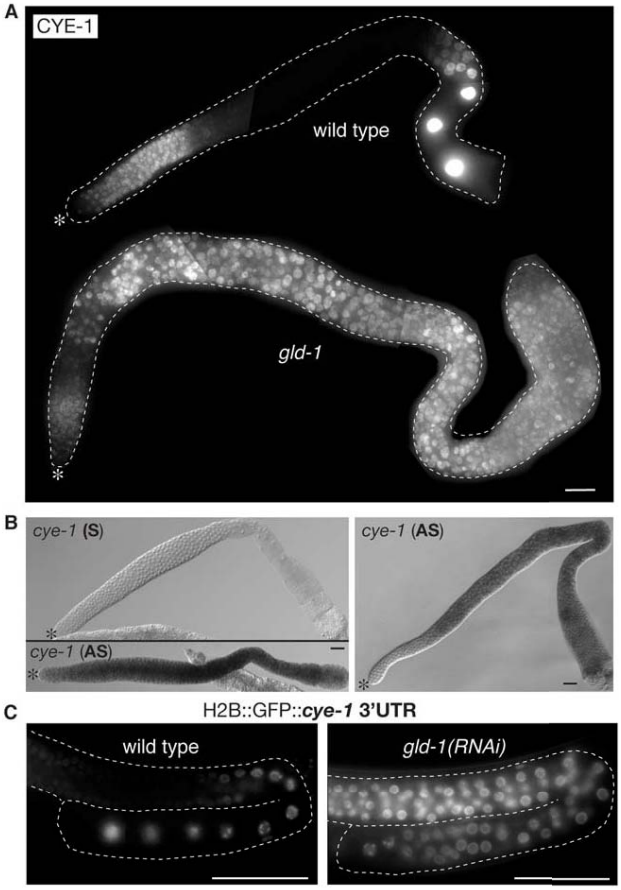


Figure S4. GLD-1 represses expression of CYE-1 through a post-transcriptional mechanism
(A) CYE-1 is de-repressed in the central gonad of *gld-1* animals. Shown are maximum intensity projections of selected optical sections of wild-type and *gld-1(q485)* gonads, extruded from about 0.5 day-old adults, that were immunostained for CYE-1. Scale bar: 20 μm.
(B) Loss of GLD-1 did not result in increased *cye-1* mRNA levels. In situ hybridization to *cye-1* mRNA on extruded gonads. The left panel shows wild-type gonads that were hybridized with either sense (S) or antisense (AS) *cye-1* probes. In the *gld-1(q485)* mutant (right panel) overall levels of *cye-1* mRNA appear lower, rather than higher, than in the wild type. Scale bars: 20 μm.
(C) The *cye-1* 3′UTR-mediated repression of the GFP::H2B reporter depends on GLD-1. The panels show GFP fluorescence micrographs of partial gonads (outlined) from young transgenic adults subjected to either mock or *gld-1* RNAi. The animals expressed the same GFP-tagged histone H2B reporter fused to the *cye-1* 3'UTR as in Fig. 5E. The control gonad shows a zone in which expression of the reporter was repressed. This repression was eliminated upon *gld-1* RNAi. Scale bar: 50 μm.

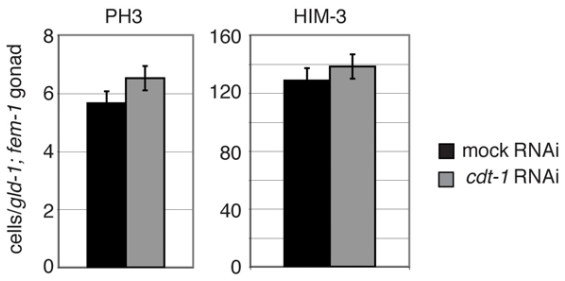
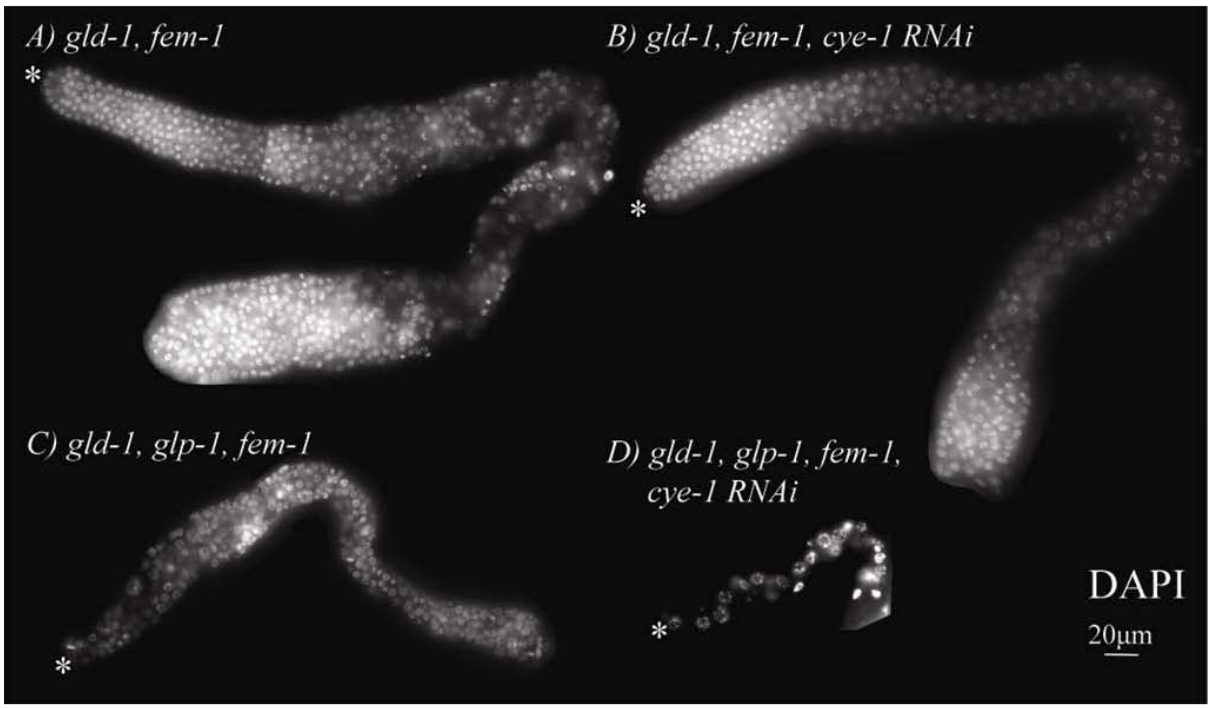


Figure S5. Depletion of CDT-1 by RNAi has little effect on the initial germ cell proliferation in the stem cell compartment or entry into meiosis in *gld-1; fem-1* gonads
In *cdt-1* and mock RNAi treated, 0.25-0.5 day-old, *gld-1; fem-1* gonads, the stem cell regions contained similar numbers of mitotic cells (determined by PH3 staining), and the gonads had similar numbers of cells in meiosis (by HIM-3 staining). Each bar indicates the mean of cells per gonad and the error bars represents the s.e.m. The numbers of scored gonads were, from left to right: 34 and 33 (PH3), 21 and 23 (HIM-3).

c. Promoting proliferation over transdifferentiation - GLP-1/Notch the second driving force in the *gld-1* tumor.

Analyzing teratoma formation in the *gld-1, fem-1* mutant showed us that the lack of translational repression of *cye*-1 through GLD-1 leads to ectopic expression of CYE-1 promoting re-entry into mitosis and cell fate change. Interestingly we observed that depletion of CYE-1 mainly arrested cell proliferation in the central region of the *gld-1, fem-1* gonad, while tumor formation still occurred in the proximal region of the gonad. In 1-1.5 day-old *gld-1, fem-1* mutants 98% of the examined gonads (n=51) had an obvious proximal bulge that contained only proliferating cells, while in the CYE-1-depleted animals such a bulge was typically smaller and was present in only 60% of the gonads (n=55) (Fig.13A-B).

Fig.13) CYE-1 and GLP-1 redundantly promote tumor formation in the gld-1, fem-1 mutant.



A-D) Gonads were stained for DAPI to visualize nuclei appearance at TP1.5.
A) In the *gld-1 fem-1* mutant tumor formation occurs throughout the proximal region of the gonad.
B) Depletion of CYE-1 prevents proliferation in the central region, while tumor formation still occurs in the most proximal region.
C) In the absence of GLP-1 tumor formation still occurs throughout the gonad.
D) Depletion of CYE-1 and GLP-1 prevents tumor formation.

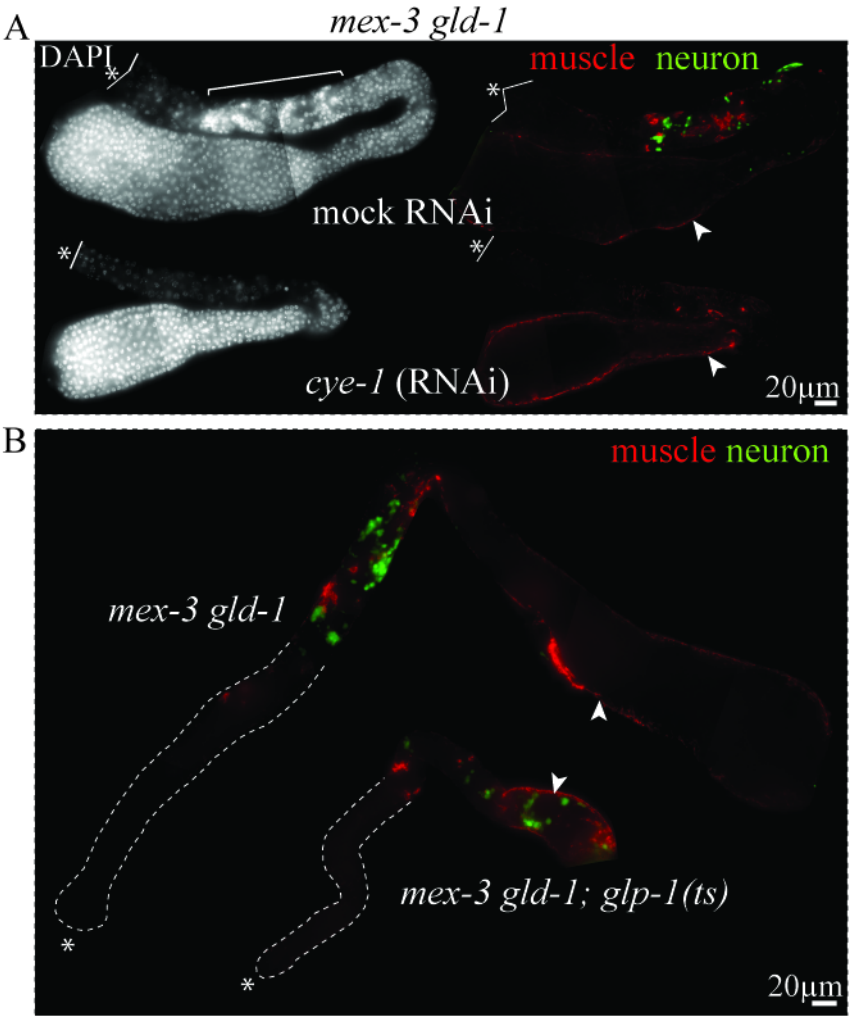
Before it had been reported that GLP-1/NOTCH, a GLD-1 target (Marin and Evans, 2003), promotes *gld*-1 tumor formation, but that GLP-1 is not essential, as tumor formation still occurs in feminized *gld-1, glp-1* animals (Fig.13 C). Feminization is required, as in *gld-1, glp-1* animals all germ cells develop as sperm (Francis et al., 1995). To achieve feminization we used the temperature-sensitive *fem-1(hc17)* mutation in our experiments.

To address the question, whether ectopic expression of CYE-1 and GLP-1 redundantly promote *gld*-1 tumor formation, we depleted both factors in the *gld-1* background. In *gld-1, glp-1, cye -1* RNAi animals we found that tumor formation was completely prevented, all cells had an enlarged nucleus, and they had ceased proliferation (average number of cells/ gonad: 35 +/- 2.2 (n=21) (Fig.13 D)). Altogether, these findings show that both, CYE-1 and GLP-1, independently promote tumor formation, while CYE-1 mainly promotes proliferation in the central region, and GLP-1 promotes proliferation in the proximal region (Fig.13).

As we had found that ectopic CYE-1 expression leads to tumor formation and transdifferentiation in the central region of the gonad, and knowing that ectopic GLP-1 signaling can prevent transdifferentiation (Ciosk et al. 2006), we wondered whether ectopic GLP-1 signaling is responsible for suppressing TD in the proximal region. To test this we used the *gld-1, mex-3* mutant background, instead of the *gld-1* mutant, as it has a higher penetrance of TD (Ciosk et al. 2006). First to confirm our finding that TD depends on ectopic expression of CYE-1, we depleted CYE-1 in the *gld-1, mex-3* mutant and assayed teratoma formation by the abundance of a neuronal-specific GFP reporter and by staining for muscle myosin. Similar to the *gld-1, fem-1* mutant, we found by DAPI staining that the central gonad of mock-treated animals contained many cells with small nuclei, but in the CYE-1-depleted animals this part of the gonad contained fewer cells which had enlarged nuclei. Importantly, in contrast to mock-treated gonads that contained many neurons and muscles, very few or no muscles or neurons were present in CYE-1-depleted *mex-3 gld-1* gonads (Fig.14A), confirming our previous finding in the *gld-1, fem-1* mutants. To test for the role of GLP-1, we constructed a *mex-3 gld-1; glp-1(2141ts)* strain, in which GLP-1 activity can be inactivated at a restrictive temperature. We found that 75% (n=28) of *mex-3, gld-1* gonads had a large proximal proliferative tumor, which in 24% contained some neurons or muscles. This is in contrast to 88% (n=32) of *mex-3 gld-1; glp-1(2141ts)* gonads which showed teratoma formation in the proximal region, but did not form a proximal proliferative tumor (Fig.14B).

This result suggests that activation of Notch signaling in the proximal tumor promotes continuous proliferation while suppressing teratoma formation.

Fig.14 Ectopic GLP-1/NOTCH promotes proliferation and suppresses transdifferentiation.



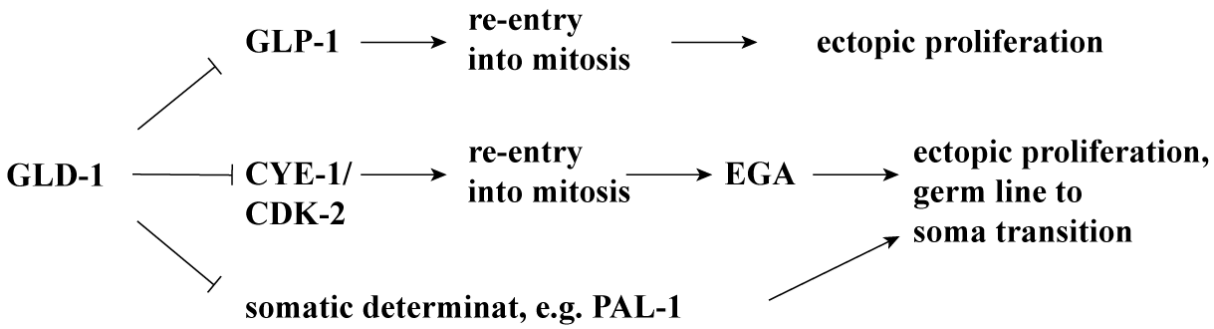
A) Teratoma formation in the central region of the *mex-3, gld-1* mutant depends on ectopic CYE-1 expression. Extruded gonads of 1 day-old animals were stained with DAPI and muscle myosin (red). The green cells express a neuronal GFP reporter. The bracket indicates the region which is filled with a heterogenous population of cells, and which contains teratomatous cells. To facilitate alignment the distal region was cropped which is indicated by the white line.
B) Loss of GLP-1 leads to teratoma formation in the proximal region. Extruded gonads of 1 day-old animals (shifted to restrictive temperature as L4) were stained with DAPI, and muscle myosin (red). The green signal shows expression of a neuronal GFP reporter.

d. Model:

i. Preventing tumor formation and germ line to soma transition – a matter of translational control of the cell cycle and differentiation factors.

Our analysis showed that ectopic expression of GLD-1 targets, such as CYE-1, GLP-1, and PAL-1, promote various aspects of the *gld-1* tumor. The *gld-1, fem-1* germ line tumor consists of two major populations of cells, central and proximal tumors, which are formed due to the ectopic expression of CYE-1 and GLP-1, respectively. Ectopic expression and activation of GLP-1 in the proximal region promotes re-entry into mitosis and leads to continuous proliferation, while maintaining germline identity and preventing germ line to soma transition. On the other hand ectopic expression of CYE-1 in the central region leads to premature activation of CDK-2, which promotes re-entry into mitosis, and embryonic gene activation. We propose that the precocious activation of an embryonic transcriptional state allows transcription factors/somatic determinants, including the GLD-1 target PAL-1, to promote germ line to soma transition.

Fig. 15) Model - GLD-1's role as tumor suppressor.



3) EXTENDED DISCUSSION

a. How could ectopic activation of CYE-1/CDK-2 promote re-entry into mitosis?

The somatic cell cycle consists of 4 sequential phase. After duplication of the genome in S-phase, cells enter the inter-G2-phase, then segregate a complete set of chromosomes to each of the two daughter cells during the M-phase, and finally enter the inter-G1-phase. In the *gld-1* mutant germ cells enter meiosis, but soon re-enter mitosis, without an additional phase of replication (Biedermann et al. 2009). As the cells in the *gld*-1 mutant enter meiosis they went through the meiotic S-phase and entered the meiotic prophase, which corresponds to the mitotic inter-G2-phase. Therefore re-entry into mitosis corresponds to the G2-M-phase transition of the mitotic cell cycle. The main regulators of the cell cycle are cyclin dependent kinases (CDKs), which become activated through their association with cyclins. In addition many more levels of regulation impinge on CDKs and cyclins, such as transcriptional control, destruction of cyclins, activating and inhibitory phosphorylations and dephosphorylations, as well as inhibitory proteins. The G2-M-phase transition is promoted by CDK-1 together with the A- and B-type cyclins. In addition to the requirement of different cyclins, CDK-1 activity is regulated by Wee1 and Myt1 kinases, two inhibitory kinases, and by the dual-specificity phosphatase CDC25, an activator phosphatase (reviewed in (van den Heuvel 2005)).

The cell cycle is tightly linked to the centrosome cycle. Centrosomes are the microtubule-organizing centers (MTOC) and are important for spindle formation during the mitotic cell cycle. They contain a pair of centrioles that duplicate once per cell cycle at the beginning of the S-phase. The centrioles undergo maturation forming two centrosomes, each of which consists of two centrioles and additional centrosomal proteins by the end of the G2-phase. At the end of the G2-phase the centrosomes undergo separation and migrate to opposite sides during mitosis, where they form mitotic spindles. At the end of mitosis/beginning of the G 1-phase centrioles disengage and the centrosome cycle begins again (reviewed in (Meraldi and Nigg 2002; Lukasiewicz and Lingle 2009)).

Different cell cycle factors are involved in the regulation of different processes throughout the centrosome cycle; for example cyclin E /CDK2 is required for centriole duplication as shown in X.laevis (Lacey et al. 1999), and $Cdc25^{string}$ is essential for centriole elongation as shown in *Drosophila* (Vidwans et al. 1999).

Although not essential for mitosis, centrosomes facilitate the timing of mitosis and function as signaling platforms, which integrate mitotic proteins (Basto and Pines 2007; Hachet et al. 2007; Portier et al. 2007). Human cell line experiments showed that several

factors promoting the G2-M phase transition are recruited to centrosomes, where they are activated at the end of G2-phase/ beginning of mitosis. Activated Aurora A kinase can be detected in centrosomes at the late G2 phase. Interestingly Aurora A is required for the recruitment of cyclin B/CDK1 to the centrosomes (Hirota et al. 2003), which become activated at centrosomes at the beginning of mitosis (Jackman et al. 2003). The activation of Cyclin B/CDK-1 depends on Aurora A, and might be facilitated through Aurora A's ability to phosphorylate CDC25B at centrosomes beginning of mitosis (Hirota et al. 2003; Cazales et al. 2005).

In the Discussion of our publication we suggest that re-entry into mitosis could be promoted through CYE-1/CDK-2 conserved function in centrosome duplication/maturation (Hinchcliffe et al. 1999; Lacey et al. 1999; Matsumoto et al. 1999; Cowan and Hyman 2006). In *C.elegans* CYE-1/CDK-2 are required for recruitment of centrosomal proteins preceding mitosis (Cowan and Hyman 2006). Among these proteins is AIR-1/Aurora A, which is essential for the timing of mitosis in embryos (Hachet et al. 2007; Portier et al. 2007). In wild type germ cells centrosomes are eliminated in maturing oocytes in the gonad (Kemp et al. 2004). In our study we found that ectopic expression of CYE-1 leads to centrosome duplication and maturation preceding re-entry into mitosis and tumor formation in the *gld-1, fem-1* mutant (Fig. 4D in (Biedermann et al. 2009)). Therefore one possible mechanism leading to re-entry into mitosis could be that ectopic CYE-1/CDK-2 activity leads to centrosome duplication and the centrosomal recruitment of mitotic factors such as AIR-1, which then initiate the cell cycle machinery promoting re-entry into mitosis.

b. How could ectopic CYE-1/CDK-2 activity promote embryonic gene activation (EGA)?

As discussed in our publication, we cannot distinguish whether ectopic CYE-1/CDK-2 directly activates embryonic gene activation (EGA), or whether it is the consequence of premature activation of the mitotic machinery. Although the fundamental mechanisms that lead to the changes in the transcriptome during EGA are emerging, very little is known about, how EGA is initiated and coordinated with oocyte maturation and the first cell divisions during embryogenesis (reviewed in (Tadros and Lipshitz 2009). Depletion of CYE-1 by RNAi leads to embryonic lethality at the 100 cell stage in *C.elegans*, which shows that CYE-1 per se is not required for early embryonic cell divisions (Fay and Han 2000). However CYE-1 could have an additional role in early embryonic stages, such as inducing EGA. One

link between the cell cycle and EGA was recently shown in mouse, where EGA first occurs in the one-cell-stage embryo (Hamatani et al. 2004). It was discovered that translation of maternally provided *cyclin A2* mRNA is required for EGA. The accumulation of Cyclin A2 leads to the activation of a CDK, presumably CDK2, and to the phosphorylation of pRB. Phosphorylation of pRB is known to modulate transcription, e.g. through the regulation of members of the E2F transcription factors. Therefore it is hypothesized that Cyclin A2/CDK-2 directly induces EGA in the one-cell-stage embryo (Hara et al. 2005). Similarly the ectopic activation of CDK-2 in the *gld-1* germ cells could activate transcription factors, and in this way directly induce EGA.

On the other hand it is also possible that EGA is initiated through a consequence of premature initiation of the cell cycle machinery. In *C.elegans* it is not known, how EGA is induced. In the 1- and 2-cell-stage embryo general RNA polymerase II dependent transcription and degradation of maternally provided proteins is blocked (Seydoux and Dunn 1997; Shirayama et al. 2006). During theses stages maternally provided RNAs and proteins are asymmetrically distributed, which leads to the establishment of a polarity within the zygote/embryo (Seydoux and Fire 1994; Mello et al. 1996; Guedes and Priess 1997; Schubert et al. 2000). The chromatin of all cells in the 2-cell and 4-cell stage embryo seems to be transcriptionally competent, as shown by H3meK4 presence (Schaner et al. 2003). Hence asymmetrically localized cell determinants are able to promote lineage formation (e.g. (Robertson et al. 2004)), as soon as the transcriptional block is relieved through an unidentified mechanism in the 4-cell stage embryo. As described in the introduction the CCCH-type zinc finger OMA-1 and -2 proteins, which are expressed by developing oocytes and are maternally provided to the embryo, play a crucial role in suppressing transcription in the 1- and 2-cell-stage embryo (Guven-Ozkan et al. 2008). Moreover OMA-1 regulates the temporal expression of maternally provided proteins, as overexpression of OMA-1 leads to stabilization and mislocalization of maternal proteins which regulate cell lineage formation (Lin 2003). Interestingly the activation of OMA-1 and -2 as transcriptional repressors and its degradation in the beginning of the 2-cell stage, is regulated through different kinases during egg activation and the first mitotic cell division (Nishi and Lin 2005; Shirayama et al. 2006). Phosphorylation of the OMA proteins by MBK-2, which is activated during the progression through the meiotic divisions (MI-MII) (Pellettieri et al. 2003; Stitzel et al. 2006; Cheng et al. 2009), is required for OMA-1 and -2 activity as transcriptional repressors (Guven-Ozkan et al. 2008). Furthermore phosphorylation by MBK-2 also primes OMA-1 and -2 for

degradation during mitosis (Shirayama et al. 2006), which is finally triggered through CDK-1 during the first embryonic cell division (Shirayama et al. 2006). Taken together the regulation of OMA proteins shows an example of how cell cycle factors coordinate progression through the cell cycle together with regulating factors required for development. This is also true for MEX-5 and MEX-6, two functional redundant CCCH finger proteins, which are required for establishing cell polarity and early cell lineage decision in the zygote and early embryo. In the early embryo MEX-5 and MEX-6 function in the activation of ubiquitin dependent degradation of germline determinants in somatic blastomeres (DeRenzo et al. 2003). The activation of MEX-5, and probably also MEX-6, depends on two kinases, PLK-1 (polo-like kinase) and MBK-2, which are both active during the meiotic division (Nishi et al. 2008). Interestingly MEX-5 and -6 are ectopically expressed in the *gld-1* mutant tumor, possibly leading to precocious degradation of germline determinants (Schubert et al. 2000; Mootz et al. 2004).

Altogether this raises the possibility that ectopic activation of the cascade of cell cycle factors, such as PLK-1, MBK-2, CDK-1, usually promoting progression through meiosis and the first mitotic cell division, leads to premature germline to soma transition through premature protein turnover of germline determinants, and the premature expression of somatic transcripts and proteins.

c. Do GLD-1 and the related quaking proteins have a conserved function in regulating development through balancing cell cycle and differentiation factors?

i. GLD-1 belongs to the STAR (signal transduction and activation of RNA) family of RNA-binding proteins.

GLD-1 belongs to the STAR (signal transduction and activation of RNA) family of RNA-binding proteins. This family is defined by a single, highly conserved, RNA binding domain of approximately 200AS, the GSG/STAR domain. This domain was initially found to be highly similar in GRP33 (brine shrimp), SAM68 (mouse), and GLD-1 (*C.elegans*), leading to its description as GSG domain (Jones and Schedl 1995). Furthermore as the activity of STAR proteins can be regulated through developmental signals (Taylor et al. 1995; Di Fruscio et al. 1999; Zhang et al. 2003b), and hence these proteins are able to link cell signaling and RNA metabolism, they are referred to as signal transduction and activation of

RNA (STAR) proteins (Vernet and Artzt 1997). Both terms are used in the literature, and both describe the same tripartite domain consisting of a maxi-KH RNA binding domain, and two flanking Qua domains. The Qua1 domain (N-terminal) is required for protein dimerization, while the Qua2 domain (C-terminal) is involved in RNA binding (Chen and Richard 1998; Ryder et al. 2004; Ryder and Williamson 2004). In addition to their RNA binding domain, several STAR proteins contain additional functional domains, such as tyrosine rich sequences, or a nuclear localization sequence (Vernet and Artzt 1997). Different mechanisms of RNA regulation, such as translational regulation, RNA stabilization/destabilization, RNA splicing, or RNA localization have been described as mode of action for different STAR proteins. And while several STAR proteins were found to function as tumor suppressors, findings in mouse, Drosophila, and *C.elegans* showed that these proteins also function as developmental regulators.

ii. GLD-1 belongs to the Quaking proteins, a STAR subfamily.

STAR proteins have been described in various organisms, such as in humans and mouse (e.g. SAM68, Quaking, SF1, SLM-1 and SLM-2), in *Drosophila* (HOW, KEP1), or in *C.elegans* (GLD-1) (Vernet and Artzt 1997; Di Fruscio et al. 1998; Di Fruscio et al. 1999; Lukong and Richard 2003; Volk et al. 2008). The STAR family consists of three subfamilies, SAM-68, SF-1, and Quaking. GLD-1 is most similar to the Quaking proteins and shows the highest identity within the STAR domain and the highest overall identity with human, mouse, Xenopous Laevis, Zebrafish, *Drosophila*, and Arabidopsis Thaliana Quaking/Quaking-related proteins (Vernet and Artzt 1997; Zorn et al. 1997) (Our bioinformatic analysis, Tab.1). A BlastP search with only the GLD-1 N- and C-terminal sequences excluding the STAR-domain showed that the similarity between GLD-1 and its homologs results from the highly conserved STAR domain.

Tab.1 Calculated identity (in percentage) of the most relevant hits of a BalstP search with GLD-1 as a query sequence.

Subfamily	Species	Protein name/ swissprot identifier	% identity within the STAR domain	% overall identity
Quaking	human	Protein quaking/ Q96PU8	52.7	29.1
	mouse	Protein quaking/ Q9QYS9	52.7	29.1
	Xenopous laevis	Protein quaking-B/ Q6IRN2	53.6	29.1
		Protein quaking-A/ Q32NN2	53.6	26.9
	Zebrafish	Protein quaking-A/ Q6P0D0	52.4	26.6
		Protein quaking-B/ Q6P104	53.0	28.2
	Drosophila	Protein held out wings/ O01367	66.8	31.6
	Caenorhabditis elegans	ASD-2/ Q65CM6	67.6	32.2
	Arabidopsis thaliana	Quaking-like protein 1/ Q0WLR1	34.5	20.6
		Quaking-like protein 3/ Q9ZVI3	42.6	17.8
		Quaking-like protein 5/ Q8GWR3	33.5	17.8
		Quaking-like protein 2/ Q9FKT4	33.5	19.2
SAM-68	human	hSLM-1/ Q5VWX1	37.4	19.6
		Sam68/ Q07666	34.5	23.3
		hSLM-2/ O75525	34.1	17.9
	Mouse	mSLM-1/ Q9WU01	37.4	18.4
		Sam68/ Q60749	34.5	23.9
		mSLM-2/ Q9R226	36.7	17.7
SF-1	human	Splicing factor 1/Q15637	39.2	14.0
	Mouse	Splicing factor 1/ Q64213	40	14.9
	Xenopous laevis	Sf1 protein/ Q7ZWT3	38.5	14.9
	Zebrafish	Sf1 protein/ B3DKQ7	39.2	15.5
	Drosophila	Splicing factor 1/ Q9VEJ1	37.7	14.5
	Caenorhabditis elegans	SF1 protein/ Q9U2U1b	37.7	16.4

A phylogenetic tree formed by STAR proteins of various organisms from human to plants, shows that GLD-1 clearly belongs to the subfamily of Quaking related proteins, which are separated from the two other subfamilies formed by SF-1 and SAM68 (Fig.16). GLD-1 falls in a group with human, mouse, Xenopouse laevis, Zebrafish Quaking, *Drosophila* HOW, and *C.elegans* ASD-2, which is in close proximity to the group of Arabidopsis thaliana Quaking like proteins.

Fig.16 Gld-1 belongs to the Quaking proteins, a STAR protein subfamily.

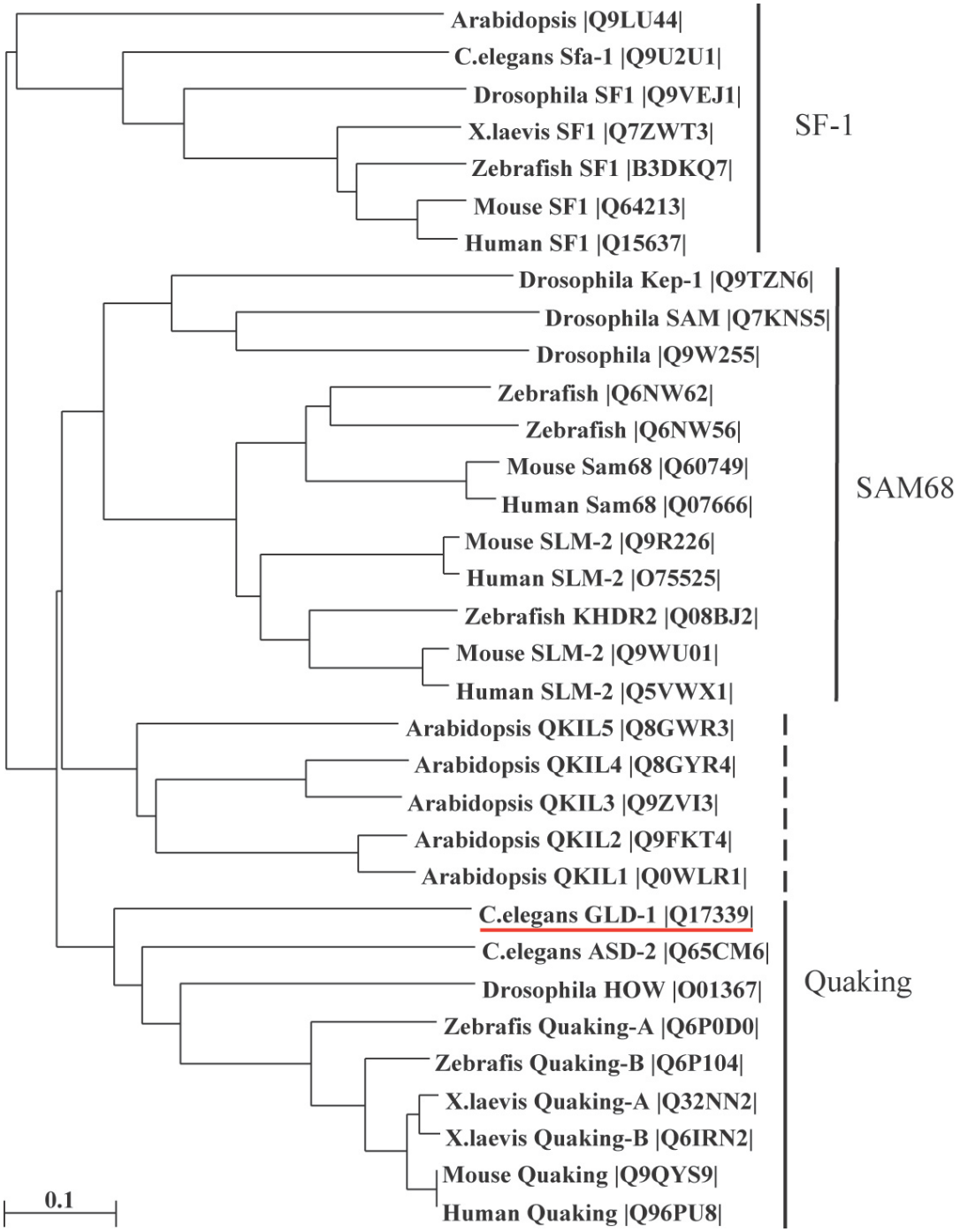


Fig.16 The most relevant hits of a NCBI BlastP (version 2.2.22) search against all human, mouse, X. Laevis, Zebrafish, *Drosophila*, *C.elegans,* Arabidopsis UniprotKB sequences (rel. 15.11) with GLD-1 (UniprotKB: Q17339) as a query sequence were aligned by using ClustalW and the tree was generated by the neighbor-joining method.

Interestingly mouse Quaking, *Drosophila* HOW, as well as *C.elegans* GLD-1 were found to regulate various developmental aspects through the regulation of cell cycle factors and differentiation factors (Li et al. 2000 ; Larocque et al. 2002 ; Nabel-Rosen et al. 2002 ; Larocque et al. 2005; Nabel-Rosen et al. 2005), which suggest that Quaking proteins might have a conserved function in regulating development through coordinating the cell cycle and differentiation factors.

iii. GLD-1, a translational regulator coordinating germ cell development in *C.elegans*.

C.elegans has two members of Quaking related proteins, ASD-2 (alternative splicing defect) and GLD-1 (defective in germline development).

The *asd-2* gene leads to the formation of two isoforms, *asd-2a*, and *asd-2b*, of which ASD-2b was found to regulate alternative splicing in body wall muscles (Ohno et al. 2008).

The second *C.elegans* Quaking related gene is *gld-1*, which produces one isoform. As mentioned in the introduction the characterization of different *gld-1* mutant phenotypes indicated that GLD-1 functions as a tumor suppressor and regulates various aspects during germ cell development in hermaphrodites, such as sentry into and progression through meiosis, the spermatogenesis/oogenesis decision, and oogenesis (Francis et al. 1995a). GLD-1 is a cytoplasm protein that shows strong expression in early meiotic germ cells (Jones et al. 1996), and in all studies so far, GLD-1 was found to function as a translational repressor. The identification of various GLD-1 targets explains GLD-1's role as a major regulator of *C.elegans* germ cell development. For example the temporal translational repression of RME-2 (yolk receptor), and OMA-1, OMA-2 (TIS11-like zinc fingers proteins), during early meiosis is important to allow proper oocyte maturation (Lee and Schedl 2001; Lee and Schedl 2004) , and translational repression of the sex determination factor TRA-2 (transformer: XX animals transformed into males, a transmembrane protein) explains GLD-1's role in regulating the sperm/oocyte fate decision in hermaphrodites (Jan et al. 1999). Interestingly, in addition to TRA-2, GLD-1 also targets another signaling protein, lin-45/Raf, which belongs to the MAP kinase pathway and regulates the pachytene to oogenesis

transition (Lee and Schedl 2001; Hsu et al. 2002). And as shown in the results section, our finding that GLD-1 represses *cye-1* in addition to *glp-1/Notch* explains its role as a tumor repressor, as well as its function in maintaining germline identity.

Taken together GLD-1 has a central role in coordinating the expression of various factors involved in cell cycle regulation and differentiation during germ cell development. This central function in cell development has also been described for other Quaking proteins, such as HOW in *Drosophila*, and the Quaking proteins in mouse and humans.

iv. How (held out wings) the *Drosophila* quaking homolog.

The *Drosophila* genome encodes 10 genes which are highly related to *quaking* (Lasko 2000). Of these homologs, *how* (held out wing) shows the highest similarity to *quaking* and is also the best characterized member of the *quaking* related genes in *Drosophila* (Fyrberg et al. 1998). The *how* gene encodes for two isoforms, the short How(S), which is encode by a 4.5kb zygotic transcript, and the long isoform How(L), which is encoded by 4.0kb maternal and zygotic transcript (Lo and Frasch 1997; Nabel-Rosen et al. 1999).

The name How (held out wings) was derived from the characteristic phenotype of hypomorphic alleles producing viable flies, which fail to fly and keep their wings extended horizontally (Zaffran et al. 1997). Analysis of various *how* mutants showed a range of phenotypes, such as defects in mesoderm, muscle, heart, tendon cell, glial cell, and imaginal disc development, as well as embryonic lethality (Baehrecke 1997; Zaffran et al. 1997; Nabel-Rosen et al. 1999; Nabel-Rosen et al. 2005; Edenfeld et al. 2006; Israeli et al. 2007).

The two isoforms, How (L) and How (S), both regulate mRNA levels through the interaction with the 3'UTR of target RNAs. However How (L) and How (S) have an opposite function. While How (L) leads to mRNA decay, How (S) stabilizes mRNAs, and can counteract How(L) mediated degradation (Nabel-Rosen et al. 1999; Nabel-Rosen et al. 2002; Israeli et al. 2007). In addition How proteins were also found to be involved in regulating alternative splicing of target genes (Edenfeld et al. 2006; Volohonsky et al. 2007).

Various factors involved in differentiation, or the cell cycle were defined as How targets, such as Cdc25/String (dual-specificity phosphatase), Stripe (EGR (early growth response)-like transcription factor), Decapentaplegic (Dpp, TGFβ homolog), Miple (heparin binding domain protein), Falten (protein with GTPase activity), Lap (ENTH domain protein), CG31638 (myosin homolog), and NeurexinIV (type I transmembrane protein) (Nabel-Rosen

et al. 1999; Nabel-Rosen et al. 2005 ; Edenfeld et al. 2006; Israeli et al. 2007 ; Toledano-Katchalski et al. 2007; Volohonsky et al. 2007).

An example of How's role in coordinating different factors to orchestrate development has been demonstrated in mesoderm development. In *how* null mutant embryos primordial mesodermal cells undergo premature cell divisions leading to a delay in mesodermal in vagination during gastrulation. This defect is rescued in *how, c dc25/string* double mutant. Consistently with the idea, that How regulates the cell cycle during mesoderm development, the mRNA levels of the mitotic activator *cdc25/string* are increased in *how* mutants, and How(L) was found to directly bind the *cdc25/string* mRNA (in vitro) and to promote *cdc25/string* mRNA degradation in cell lines (Nabel-Rosen et al. 2005). This finding suggests that HOW(L) functions as a repressor of *cdc25/string* to facilitate a cell cycle arrest, which precedes and is necessary for proper mesoderm invagination (Nabel-Rosen et al. 2005). Mesoderm invagination is followed by mesoderm spreading. During this process mesodermal cells spread over the ectoderm. *How* mutants are also defective in mesoderm spreading. Three direct How(L) targets were found to be upregulate in the mesoderm of *how* germline clone mutant embryos (*miple, falten*, *CG31638*), and its ectopic expression was found to lead to mesoderm spreading defects. However while the molecular mechanism leading to mesoderm spreading defects is not understood, it is interesting to note that ectopic expression of *miple* leads to ectopic activation of the MAP kinase pathway, which is also observed in the *how* mutants. This finding might explain the mesodermal spreading defect and further shows an example of how Quaking related proteins can impinge on conserved signaling pathways through RNA regulation (Toledano-Katchalski et al. 2007).

As mentioned in the beginning How(S) can counteract HOW(L) repression. This opposite function of the two How isoforms is critical for the differentiation of tendon precursor cells to mature tendon cells (Nabel-Rosen et al. 1999; Nabel-Rosen et al. 2002) Ectodermal derived tendon cells are required for the attachment of muscle cells to the exoskeleton. The interaction between muscle and tendon cells is essential for proper development of both cells types (reviewed in (Volk 1999)). How(L) and How(S) were found to regulate the temporal expression of Stripe, an EGR (early growth response) -like transcription factor, and a key regulator of tendon cell differentiation. In vivo and cell line experiments showed that How(L) and How(S) regulate Stripe levels through mRNA decay, or stabilization, respectively, and this function is mediated by the *stripe* 3'UTR (Nabel-Rosen

et al. 1999 ; Nabel-Rosen et al. 2002) . This shows an example of how Quaking related proteins can control the temporal expression of a differentiation factor.

v. The mouse Quaking proteins

The mouse homolog of How was identified through the analysis of a spontaneous mouse mutant (*quaking*^{viable}) that showed tremor and strong myelination defects in the central and peripheral nervous system (CNS and PNS) (Sidman et al. 1964; Ebersole et al. 1996). Moreover the isolation of different embryonic lethal mutations in *quaking* showed a variety of developmental defects and the requirement of Quaking proteins in early embryogenesis before the establishment of a functional nervous system (Justice and Bode 1988; Cox et al. 1999). Similar to *Drosophila*, the mouse *quaking* gene encodes for different isoforms. Through alternative splicing six different transcripts are formed, 5 kb-A, B, 6 kb, 7 kb-A, -B and qkI $^{\Delta KH}$. These isoforms are different by their carboxy-terminal sequence and their 3'UTR, but all contain the STAR domain sequence (except the qkI$^{\Delta KH}$) (Kondo et al. 1999). Three QKI proteins, QKI-5 (encoded by the 5kb-A transcripts),QKI -6 (encoded by the 5kb-B and 6kb transcripts), and QKI-7 (encoded by the 7kb-A transcripts) are described (Hardy et al. 1996).

The molecular function of Quaking proteins is only partially understood and includes RNA localization (Larocque et al. 2002), regulation of alternative splicing (Wu et al. 2002), as well as RNA stabilization (Larocque et al. 2005).

Analysis of the hypomyelination phenotype in the *quaking*^{viable} mice showed that Quaking proteins play a fundamental role in oligodendrocyte differentiation through the regulation of various targets, such as P27 (CDK inhibitor), MAG (myelin-associated glycoprotein), and MBP (Myelin Basic Protein).

The Myelin Basic Protein (MBP) is a myelin component that is required for myelin assembly (Simons and Trotter 2007), and in mouse four MBP isoforms (21.5, 18.5, 17.2, and 14 kDa) are produced via alternative splicing (de Ferra et al. 1985). In *quaking*^{viable} mice brain three MBP mRNA isoforms (18.5, 17.2, and 14), as well as all MBP protein isoforms (21.5, 18.5, 17.2, 14kDa) are reduced during postnatal development. *mbp* transcription, as well as *mbp* translation, are normal, however cytosolic *mbp* mRNA levels are reduced and the cellular *mbp* mRNA localization is altered in *quaking*^{viable} mouse brain (Li et al. 2000).

In oligodendrocytes and Schwann cells, the myelin producing cells, QKI-5 is mainly expressed in the nucleus, due to a novel nuclear localization sequence. On the other hand

QKI-6 and QKI-7, which lack this nuclear localization sequence, are primarily expressed in the perikaeryal cytoplasm (Hardy et al. 1996; Wu et al. 1999). Overexpression of QKI-5 in cell lines leads to the loss of *mbp* mRNA and protein in the processes of oligodendrocytes and to the restriction of *mbp* mRNA and protein to the nucleus and to the perikaryon. Similar in vivo overexpression of QKI-5 leads to loss of MBP protein in oligodendrocyte in the brain, while overexpression of QKI-6, and/or QKI-7 promotes MBP expression (Larocque et al. 2002; Larocque et al. 2005). Consistently in *quaking*^{viable} mice, which lack QKI-6 and QKI-7 expression in oligodendrocytes, *mbp* mRNAs are strongly reduced in myelin membrane fractions and are retained in membrane free polyribsome fractions of *quaking*^{viable} mice brain samples (Li et al. 2000). Furthermore no *mbp* mRNAs can be detected along oligodendrocyte axons in *quaking*^{viable} mice (Larocque et al. 2002). Importantly overexpression of QKI-6 in *quaking*^{viable} mice restores *mbp* mRNA and protein expression, and rescues the myelination defect, as well as the tremor phenotype (Zhao et al. 2006). These experiments show an opposite function of Quaking isoforms and cell line experiments suggest that the interplay between all three Quaking isoforms is necessary to fine tune proper MBP expression (Larocque et al. 2002).

Taken together, although the molecular mechanism of how Quaking proteins regulate MBP protein expression is not completely understood, a major function of Quaking proteins seem to lie in the stabilization of cytosolic *mbp* mRNAs, as well as in the localization of *mbp* mRNAs to the periphery of myelinating cells, to facilitate the proper incorporation of MBP proteins into myelin sheaths.

In addition to its role in myelination, Quakings also function in the cell fate decision between neuro, and glia cells. Quaking proteins are specifically expressed in neuronal progenitor cells which acquired the characteristics of glia cell progenitors during embryogenesis and postnatal development (Hardy 1998). Retroviral expression of QKI-6 and QKI-7 in multipotential neuronal progenitor cells during embryogenesis drives the majority of Quaking expressing cells into glia fate, oligodendrocytes and astrocytes, demonstration Quakings potential in promoting glia cell fate determination. Moreover studies in rat oligodendrocytes cultures showed that ectopic expression of QKI-6 and -7 leads to G_o/G_1 arrest and to an enhancement of oligodendrocyte maturation. QKI-6 and -7 were found to directly bind to and to stabilize p27 mRNA in oligodendrocyte cultures. In this way Quaking proteins are able to directly regulate a target that is involved in both cell cycle control and

oligodendrocyte differentiation (Casaccia-Bonnefil et al. 1997; Tokumoto et al. 2002; Larocque et al. 2005).

Similar in primary rat co-cultures of Schwann cells and neurons, ectopic expression of QKI-6 and/or QKI-7 leads cell cycle arrest, to ectopic expression of P27, and to the expression of the Schwann cell differentiation marker MBP. On the other hand, downregulation of *qki* by siRNA leads to strong reduction of mRNA levels encoding for *mbp, p27* and *krox 20*, a transcription factor that is critical for PNS myelination, demonstrating also here a role for Quakings to coordinate the differentiation processe (Larocque et al. 2009).

vi. Different mechanisms of RNA regulation, but balancing cell cycle and differentiation factors seems to be a conserved function of Quaking homologs.

Taken together different mechanisms of RNA regulation, such as translational regulation, RNA stabilization/destabilization, or RNA localization have been described for the different Quaking homologs. However whether the different Quaking homologs have adapted specific roles in RNA regulation in different tissues and organism during evolution, or whether these differences only reflect our incomplete understanding of their molecular function, and possibly Quaking proteins posses the properties of general RNA regulators, which control many aspects of RNA regulation, cannot be said yet, as the molecular functions of Quaking and Quaking related proteins are just being about to be resolved.

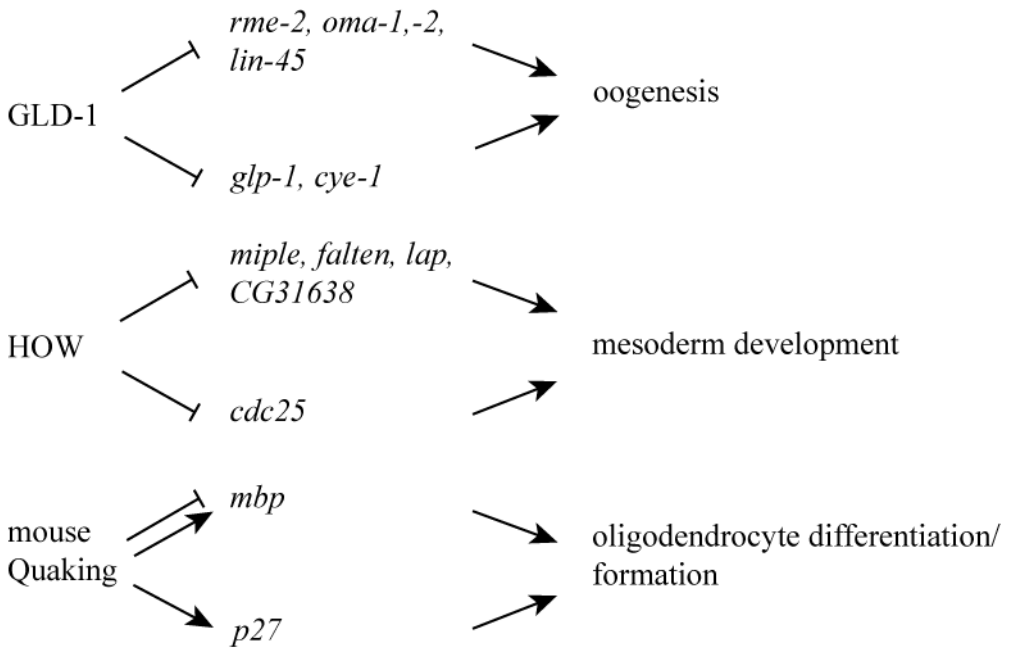
For all the species discussed, different examples were described, that show that Quaking and Quaking related proteins are involved in the regulation of cell development through the regulation of cell cycle and differentiation factors (Fig.17).

In mouse Quaking proteins were found to orchestrate the expression of various factors involved in oligodendrocyte and Schwann cell differentiation. Such as p27, which is involved in the regulation of the cell cycle withdrawal and in the actual oligodendrocyte differentiation process (Casaccia-Bonnefil et al. 1997), as well as several other factors that are involved in myelin formation.

In Drosophila How is required for the temporal control of mesoderm development. First How is needed to arrest cell division, to allow proper mesoderm invagination, and second How is required to repress various maternal and zygotic mRNAs to allow proper mesoderm spreading.

And finally in *C.elegans* GLD-1 is required to prevent ectopic expression of *cye-1/cyclin E* and *glp-1/Notch* to maintain mitotic quiescence during meiosis, and it is also required to regulate the expression of factors involved in oogenesis.

Fig.17 The dual function of Quaking and Quaking related proteins in regulating the cell cycle and differentiation factors to orchestrate development.



vii. Quaking's function as tumor suppressor.

Due to their central function Quaking proteins seem also to function as tumor suppressors. As already described, GLD-1 functions as a tumor suppressor in the *C.elegans* germline, and mutations in human *quaking,* and alterations in human *quaking* expression, were found to correlate with tumor formation (Li et al. 2002 b; Ichimura et al. 2006 ; Mulholland et al. 2006). A recent study showed that QKI-5 and -6 were greatly reduced in human colon cancer cells in comparison to the adjacent normal epithelial cells. Ectopic expression of QKI-5 and -6 in HT29 colon cell line cells leads to the expression of various differentiation markers, and QKI-5 and -6 were found to stabilize *p27* mRNA, and to negatively regulate *β-catanin* activity in HT29 cells. This result suggests, that Quaking proteins balance the cell cycle and differentiation in a similar way as described before in colon epithelia cells, and it suggests a function of Quaking proteins as tumor suppressors (Yang et al. 2009).

viii. Balancing cell cycle and differentiation factors is a central function to regulate development.

In *C.elegans* loss of GLD-1 leads to ectopic expression of Cyclin-E, which together with CKD-2 promotes the re-entry into mitosis in meiotic *C.elegans* germ cells. Re-entry into mitosis then leads to embryonic gene activation (EGA), and transdifferentiation. In general during development stem cells and progenitor cells have the ability to undergo self-renewal and, or to differentiate into lineage specific cell types. Central to self-renewal and cell lineage decision is a complex network of cell cycle factors and differentiation factors which underlie mutual regulation. This has been extensively studied during neurogenesis (reviewed in (Cremisi et al. 2003; Ohnuma and Harris 2003)). Different cell cycle factors were found to regulate neuronal fate choice independent of their function regulating the cell cycle. For example P27 was found to promote the decision of neuroblast cells to acquire a neuronal fate through the stabilization of the helix-loop-helix transcription factor neurogenin in X.laevis (Vernon et al. 2003). Or Cyclin E was found to represses the transcription factor Prospero and to promote stem cell identity in *Drosophila* neuroblasts (Berger et al. 2009). Prospero on the other hand is a cell-fate determinant that regulates various genes involved in neuroblast self-renewal, differentiation, and the cell cycle on a transcriptional level (Choksi et al. 2006). Due to its function in regulating both the cell cycle and differentiation, Propero has been described as a dual function molecule. Some more dual function molecule proteins were described, e. g. Geminin which coordinates proliferation and differentiation through its various effects on regulating transcription (reviewed in (Seo and Kroll 2006)). The fundamental importance of transcriptional regulation during development has been well described. However regulation of development is not restricted to transcription, as many more levels of regulation are involved, such as RNA regulation, as it has been show for the regulation of heterochronic genes in *C.elegans* (Moss 2007).

Therefore various mechanisms are involved in the mutual regulation of differentiation and cell cycle factors. Disturbing this balance can lead to various developmental defects, such as defects in oogensis, or tumor formation in *gld-1* mutants. In this way Quaking proteins can be added to the group of dual function molecules due to their described function to orchestrate development through the regulation of differentiation and cell cycle factors, and furthermore they extend the complexity of the regulatory network through their function as RNA regulators.

4) SUMMARY

The fertilization of an oocyte with sperm leads to the formation of a zygote, which has the unique ability to differentiate into any cell type. This specific ability is defined as totipotency. Germ cells differentiate into highly specialized cells, oocytes and sperm, but germ cells also have an underlying totipotency, as totipotent cells can be derived from germ cells. However the mechanisms that allow germ cells to establish/maintain germline identity and to specialize, while maintaining an underlying totipotency, are not understood.

In *C.elegans,* germ cells in the *gld-1,* and *gld-1, mex-3* mutants fail to progress through meiosis and instead form a germline tumor. Recently Dr. Rafal Ciosk found that germ cells in the *gld-1,* and *gld-1, mex-3* germline tumor lose their germline identity and instead acquired a somatic fate, a phenotype that is reminiscent to a special human germline tumor, called teratoma. This finding provided us with a genetic model system that allowed us to investigate the mechanisms that are required to maintain germline identity, and totipotency.

To address these questions, we first needed to understand how teratoma formation occurs in *C.elegans*. What is the etiology of the cells undergoing teratoma formation? To address this question we used a compound mutant background in which the major mitotic and meiotic pathways were deleted and the gonad was lacking a distal to proximal orientation. As cells within this gonad showed a synchronized development we could follow the different cell cycle stages preceding teratoma formation. After an initial phase of proliferation germ cells enter meiosis, however fail to progress through meiosis, re-enter proliferation and undergo germ line to soma transition. This knowledge allowed us to reveal the cells that lead to teratoma formation in the simplest genetic background, the *gld-1* mutant. This analysis showed us that the germline tumor in the *gld-1* mutant is formed by two major populations of cells, a central and proximal tumor. As already the loss of GLD-1 alone leads to teratoma formation we sought to identify GLD-1 targets. In this analysis we could define core cell cycle factors as new GLD-1 targets, namely cyclin E and Cyclin Bs. Genetic experiments showed that ectopic expression of Cyclin E together with CDK-2 promotes the re-entry into mitosis and tumor initiation in the central region of the *gld-1* gonad. This re-entry into mitosis leads to loss of germ line identity and unexpectedly to a change in the transcriptional program of the cells, preceding expression of markers of terminally differentiated cells. Furthermore we found that ectopic expression of a known GLD-1 target, GLP-1, promotes proximal tumor formation and suppresses germ line to soma transition in these cells.

Taken together this study revealed that different cell populations lead to the formation of the heterogeneous germline tumor in the *gld-1*, or *gld-1, mex-3* mutant, and identified its

major regulators. Further this study provides a first mechanism promoting germline to soma transition. We propose that the loss of GLD-1 leads to ectopic expression of its targets, such as Cyclin E and the somatic determinant PAL-1/Caudal. Ectopic expression of Cyclin E promotes re-entry into mitosis and a change in the transcriptional profile of the cell, which creates an environment that allows a somatic determinant to promote germ line to soma transition. The importance of this finding is that it is not only the loss of translational control that leads to teratoma formation, but also a change in the transcriptional competence of the cells, and it emphasizes the importance of cell cycle control during meiosis as a fundamental mechanism to maintain germline identity.

5) ADDITIONAL MATERIALS AND METHODS

a. Additional worm strains:

Following additional worms strains were used:

- gld-2(q497) gl d-1(q495); u nc-32(e189) gl p-1/hT2 (qIs48; ph aryngeal G FP), [unc119::GFP, rol-6 (su1006)] > lab ID: 48
- mex-3(or20) gl d-2(q497) g ld-1(q485), un c32(e189) gl p-1 (q175)/hT2 (qIs48 pharyngeal GFP), [unc-119::GFP, rol-6 (su1006)] > lab ID: 99
- gld-2 (q497) g ld-1 (q485); u nc-32 (e189) g lp-1 (q175)/hT2 (qIs48; p haryngeal GFP+); integrated myo-3::YFP (Fire vector L 4671, pPD133.63) > lab ID: 45
- mex-3 (or20) gld-2 (q497) gld-1 (q485); unc-32 (e189) glp-1/hT2 (qIs48; pharyngeal GFP); integrated myo-3::YFP (Fire vector L 4671, pPD133.63) > lab ID: 51
- gld-1 (q485); unc-32 (e189) glp-1 (q175)/ hT2[qIs48]; fem-1 (hc17ts) > lab ID: 120
- gld-1(q485)/ hT2[qIs48]; fem-1 (hc17ts) (CGC: RAF-3) > lab ID: 121
- mex-3(or20)/hT2[qIs48], unc-119::GFP > lab ID: 1
- mex-3 (or20) gld-1 (q485); g lp-1 ts (e2141); h im-8 (e1489)/ hT2 (qIs48; pharyngeal GFP); edIsb [unc119::GFP, rol-6 (su1006)] X > lab ID: 180
- mex-3 (or20) gld-1 (q485)/ hT2 (qIs48; pharyngeal GFP); edIsb [unc119::GFP, rol-6 (su1006)] X > lab ID: 14

b. Creation of transgenic lines

i. Injection.

Microinjection w as p erformed as d escribed in wormbook (http://www.wormbook.org/chapters/www_transformationmicroinjection/transformationmicroinjection.html). T he bacterial c lone car rying the Fire expression vector (pPD133.63) containing a Y FP r eporter u nder the co ntrol o f t he muscle myo-3 pr omoter (http://www.addgene.org/docs/fire/andrew/Vec99.pdf), w as g rown in a 2 00ml LB medium culture over night at 37°C and the vector was purified the next day using the Midi-Qiagen kit (Cat. No. 12143). The purified DNA was diluted at a concentration of 100ng/µl in H_2O and injected with a pressure of 6094h Pain into the gonads of adult wild type worms by using a Zeiss A xiovert200M equipped w ith a E ppendorf micro manipulator/ T ransjector 5246. The injection needles were pulled by Jacqueline Ferralli using a N eedle/Pipette Puller Model 720 from D avid Kopf I nstruments T ujunga C alifornia U SA. T he settings w ere, s olenoid: 1. 8, heater: 10.8.

ii. Integration of extra chromosomal arrays.

40 L4 animals were transferred on a NG2% plate, seeded with OP50 bacteria. The plate was irradiated with 2500 rads (386s, 120V). The worms were cultured for 2d at 20°C and ~500 F1 were singled on a new plates. After 3d 4-8 F2 worms were singled from plates that showed a very high transmission rate, and the following F3 generation was scored for 100% transmission rate. The expression pattern of favorite clones was validated, and the worms were backcrossed to the wild type N2 strain.

iii. Bombardment protocol.

1. Worm culture.

unc-119 (ed3) worms were starved on 1-2 large (~15cm) peptone rich plates (2.5% w/v Difco-Agar (BD 214530), 2% w/v Bacto-Peptone (BD 211677/BD 211820), 0.12% w/v NaCL, 5µg/ml cholesterol, 1 mmol/l $MgSO_4$ 25mmol/l KH_2PO_4), which were seeded with NA22 E.coli bacteria, at 20°C. The worms were transferred on 6-8 large NA22 peptone rich plates, grown till adult stage, bleached, and synchronized on plates without food (Dauer plates) at 20°C. The larvae were distributed on large NA22 peptone rich plates, cultured at 20°C, and used for bombardment as L4/young adults.

2. Gold particle preparation.

25mg of gold particles (Chanpur, Karlsruhe, Cat. No. 009150, 0.3-3 micron) were weighed into a siliconized 1.5ml tube. 1 ml of 70% Ethanol was added, the particles were agitated for 5min on a vortex machine, and the tube was put in a rack for 5min to allow the beads to settle. After a short spin with the table centrifuge, the Ethanol was removed and the same procedure was repeated 3 times with sterile distilled water. After the last washing step, the water was removed and the beads were resuspended in sterile 50% glycerol (in ddH_2O). The beads can be stored for 4 weeks at 4°C.

To prepare the micro carrier, they were soaked in 100% isopropanol (Merck), dried, and inserted into the bombardment holder. In parallel the worms were collected in M9 buffer, washes, and plated in the center of two 10cm NG2% agar plates.

To load DNA on the gold particles, beads were agitated for 5 min on the vortex machine, 80µl were removed, and transferred into a siliconized 1.5ml tube. The beads were mixed on a vortex again for 3min, and immediately 32µl of 0.1M spermindine was added. The beads were agitated for 1min, and 8µl of DNA (0.5M) were added. Subsequently 80µl of 2.5M $CaCl2_2$ were added drop wise, while the beads were constantly agitated on the vortex

machine. The tube was put in the rack to allow the beads to settle for 3min, the supernatant was removed, and the gold particles were resuspended in 240µl 70% Ethnaol. As the gold particles are sticky to the wall of the tube, it is necessary to scratch them of the wall with the pipette tip. Finally the particle were spinned down for a few seconds, and resuspended in 80µl 100% Ethanol. The gold particles were resuspended by pipetting, and mixed by using a vortex for 3min. 10µl were loaded onto one microcarrier. The Biolbalistic Particle Delivery Sytem (BioRad Model PDS-1000) was used for bombardment with a pressure of 1350psi. And the worms were shot twice per DNA construct.

After shooting the worms were kept at 15°C for 2h, then distributed on 5cm NG2% agar plates, seeded with OP-50 E.Coli bacteria, and cultured at 25°C. After 2-4 weeks wild type moving worms were singled on separated plates.

c. In situ hybridization.

i. In situ probe preparation

The in situ hybridization was basically performed as described byGina Broitman-Maduro and Morris F. Maduro (http://www.faculty.ucr.edu/~mmaduro/resources.htm). Total RNA was extracted from ~500µl wild type worm pellet according to the Trizol protocol (Invitrogen Cat. No. 15596-026), DNA contamination was removed by using the Ambion DNA-free kit (AM1906), and cDNA was synthesized from 1.2g RNA using oligo dT primers and the Im Prom-II kit from Promega (A3800). Two cDNA reactions (each 20µl) were pooled and supplemented with 60µl DEPC H_2O. 5-10µl cDNA were used in a 100µl PCR reactions to produce the probe template DNA (Fast Start PCR, Roche, 12 032 929 001). The right length PCR product was gel purified (Promega Wizard SV Gel and PCR Clean-Up System, A9281) and additionally purified by a standard Ethanol precipitation. The pellet was dissolved in 20µl DEPC H_2O. 1µg of the purified PCR product was used for DIG RNA labeling according the standard protocol, including the DNAse I digest (DIG RNA labeling Kit (SP6/T7) Roche, Cat. No. 11 175 025 910). For purification, 2.5µl LiCl (4M), and 75µl 100% ice cold ethanol were added, the probe was incubated at -80°C for 1h, centrifuged at 13000g for 15min at 4C°, and the supernatant was decanted. Then the probe was washed in 50µl 70% ice cold ethanol, centrifuged for 5 min at 4 °C, the supernatant was decanted and the pellet was air dried at room temperature. Finally the probe was resuspended in 50µl DEPC H_2O, subdivided into 5µl aliquots, and stored at -20°C.

To check for probe quality, it was run in a 2% agarose gel, and the labeling efficiency of different probes was estimated by performing a dot plot as described in DIG Northern Starter Kit protocol (Roche, Cat. No. 12 039 672 910).

ii. Gonad dissection, fixation, and hybridization.

Gonads were dissected in 40µl M9/Levamisol (c=1mM) buffer on a microscope cover slip. Most of the liquid was removed, the cover slip with the worms was placed on a poly-L-Lysin coated microscopy slide, and the slide was frozen on dry ice. For fixation the cover slip was flipped off the microscopy slide and the sample was incubated in 100% Methanol (-20°C) for 5min. This was followed by a hydration series, consisting of 90%, 70%, 50%, and DEPC-H_2O; the sample was incubated for 5min at each step. The sample was then fixed in NTF (protocols for buffers and solutions, see original Maduro protocol) at 37°C for 1h (jar was kept in the waterbath). Afterwards the sample was rinsed twice in DEPC-H_2O, and twice in 2 xSSC, each time for 5 min at room temperature. The slides were placed in a humidity chamber, and a 300µl drop of prehybridization buffer was added to the sample, the slides were incubated for 1 h at 4 2°C. The probe was diluted at a concentration of 1ng/µl in prehybridization buffer, heated at 65°C for 5min, and then chilled at room temperature. 60µl probe were added per slide, which was then covered by a microscopy slide and sealed with rubber glue. The probe was hybridized overnight at 4 2°C. The next day the slides were washed two times in 2xSSC, and two times in Formamide buffer (FB), each time 5 min at 42°C. This was followed by a second round of washes, two times in 2 xSSC, in Tris-NaCl, and then in TN buffer, each time the washes were done for 5min at room temperature. A jar containing blocking buffer was kept in the waterbath at 37°C and the sample was incubated for 3 0min. The anti-DIG antibody was diluted 1:3000 in blocking buffer and 50µl were applied per slide. The slides were covered with a glass slide, put in a humidity camber and incubated for 3h at 37°C. Afterwards the slides were washed two times in TN buffer, and one time in TNM buffer for 1 0min each time. Then the slides were put in a jar containing developer solution for approximately 4 h. After 2h the positive and negative controls were regularly controlled for signal development. At the time the sample showed a clear signal in the positive control, the reaction was stopped by washing the slides in TN-EDTA. Finally the samples were mounted with Vectashield (Vetor S1024).

iii. Image acquisition.

The images were captured with a Zeiss ImagerZ1 microscope equipped with a 63x/1.4 oil lense and AxiocamMRm (Zeiss) camera. Images were acquired with the same exposure time and processed in Adobe Photoshop CS3 in an identical manner.

In situ primer sequences:

Primer name	Primer sequence	Primer target sequences and orientation
BB61	gaagaatacaaggaaagaatgactg	vet-1-AS fw
BB62	TAATACGACTCACTATAGGGACcattttggagtgtttccttgatagc	vet-1-AS rev
BB63	TAATACGACTCACTATAGGGACgaagaatacaaggaaagaatgactg	vet-1-SENSE fw
BB64	cattttggagtgtttccttgatagc	vet-1-SENSE rev
BB53	aatccgaatctgcttacgaatctg	vet-2-AS fw
BB54	TAATACGACTCACTATAGGGACTttaacacctccaaatggtccgcc	vet-2-AS rev
BB55	TAATACGACTCACTATAGGGACaatccgaatctgcttacgaatctg	vet-2-SENSE fw
BB56	ttaacacctccaaatggtccgcc	vet-2 SENSE rev
BB33	TAATACGACTCACTATAGGGACTtctcttttcattgtggtagcgtcg	vet-4-AS rev
BB34	ttcatctacacccttgggctcgg	vet-4-AS-primer fw
BB35	tctcttttcattgtggtagcgtcg	vet-4 -Sense rev
BB36	TAATACGACTCACTATAGGGACTttcatctacacccttgggctcgg	vet-4 -Sense fw
BB49	catatggttcgccgttcattcgc	vet-6-AS fw
BB50	TAATACGACTCACTATAGGGACTatctctgcatactttgaagcacgc	vet-6-AS rev
BB51	TAATACGACTCACTATAGGGACTcatatggttcgccgttcattcgc	vet-6-SENSE fw
BB52	atctctgcatactttgaagcacgc	vet-6-SENSE rev
BB29	attatctcagatgttgatgcgatgc	pes-10-AS fw
BB30	TAATACGACTCACTATAGGGACTcaagttgtgcagcaagtcctgattc	pes-10-AS rev
BB31	TAATACGACTCACTATAGGGACTattatctcagatgttgatgcgatgc	pes-10-SENSE fw
BB32	caagttgtgcagcaagtcctgattc	pes-10-SENSE rev
BB37	TAATACGACTCACTATAGGGACTtggcgtaatccttgacaataactcg	nhr-2-AS rev
BB38	tggtttgtggtgataactctactgg	nhr-2-AS fw
BB39	tggcgtaatccttgacaataactcg	nhr-2 -Sense rev
BB40	TAATACGACTCACTATAGGGACTtggtttgtggtgataactctactgg	nhr-2-Sense fw
BB45	TAATACGACTCACTATAGGGACTagagtttcgccgacgcagatcg	hlh-1-AS rev
BB46	tgcacctaccactttctactcgg	hlh-1-AS fw
BB47	agagtttcgccgacgcagatcg	hlh-1-SENSE rev
BB48	TAATACGACTCACTATAGGGACTtgcacctaccactttctactcgg	hlh-1-SENSE fw

d. DNA fluorescent in situ hybridization (FISH)

The DNA was performed according to a protocol from Györgyi Csankovszki from the Barbara Meyer laboratory (UC Berkeley, USA).

i. Probe labeling and purification.

The probe was labeled according to the Nick Translation Mix protocol (Roche, Cat.No. 11 745 808 910) and purified using the Promega Wizard SV Gel and PCR Clean-up system. 40µl salmon sperm (10mg/ml) were added and the DNA was additionally purified by a standard ethanol precipitation. The pellet was resuspended in 160µl deonized formamide (Merck) through vigorous pipetting. The probe was denatured at 72° for 10 min, chilled on ice, resuspended in 160µl 2xhyb buffer (1 part 20xSSC, 2 parts 10mg/ml BSA, 2 parts 50% dextran sulfate), and was stored light protected at –20°C.

Probe templates:

- *baf-1:* cosmid containing the *baf-1* coding sequence (wormbase: B0464)
- *5srRNA*: the template DNA was PCR amplified from wild type N2 genomic DNA and gel purified.

  Primer sequences:

  SG-2185: TACTTGGATCGGAGACGGCC

  SG-2186: CTAACTGGACTCAACGTTGC

ii. Gonad dissection, fixation, and probe hybridization.

Gonads were dissected in 40µl sperm salt (50mM PIPES ph =7 (dissolves only completely, if ph is adjusted), 25mM KCL, 1 mM $MgSo_4$, 45mM NaCl, 2 mM $CaCl_2$), most liquid was removed, gonads were fixed in 8µl 1% PFA/ sperm salt buffer for 5min at RT, and transferred on a poly-L-Lysin coated slide. The slide was frozen on dry ice, after at least 5min the sample was cracked open and the slide was dehydrated in a series of Ethanol dilutions, 70% (-20°C), 80% (RT), 95% (RT), 100% (RT) for 2min each. The sample was air dried and 20µl in situ probe were applied. For denaturation, the slide was placed on a 95°C heat block for 3 min, and then put in a humidity chamber at 37°C over night. The next day the slides were washed 3 x5min in 2 xSSC/ 50% formamide (39°C), 3x 5min in 2 xSSC (39°C), and 1x10min in 1 xSSC (39°C). For DNA staining the slides were placed in a jar containing 4xSSC and Hoechst 34580 (1:10000, Invitrogen H 21486) for 10 min at RT. This was

followed by 3 x5min washes in 2 xSSC and the slides were mounted in Prolong gold (Invitrogen P36930).

iii. Image acquisition.

Images were acquired with a Zeiss Confocal LSM510 META, Axioplan2 microscope, deconvolved by using the Huygens Remote Manager (Ponti A. et all. 2007). Imaris software was used for image analysis.

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