Control of pluripotency during the oocyte-to-embryo transition in *Caenorhabditis elegans*

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Summary

The germline of *Caenorhabditis elegans* has been used as a model system to study the control of pluripotency in germ cells: through the mean of a genetic screen, from which most of the results of this thesis derived, different factors involved in the control of pluripotency in growing oocytes have been found. It is interesting to note that all the factors identified in the screen are cytoplasmic RNA-binding proteins (RBPs) involved in different aspects of post-transcriptional gene regulation. Post-transcriptional gene regulation can therefore be considered the main mechanism through which a "quiescent" pluripotent state is maintained in oocytes until after fertilization. The oocyte-to-embryo transition has been shown to occur in the absence of polymerase II-dependent transcription, a fact which better clarifies the general importance of RBPs in this developmental context. This study has been the first one to provide the example of a factor, LIN-41, which regulates pluripotency specifically in developing oocytes. *lin-41* mutant oocytes lose their germline identity, enter an embryonic and pluripotent state and terminally differentiate into somatic cells. Although LIN-41 was already known in *C. elegans* to be involved in a somatic developmental pathway, it appears to regulate different targets in the two developmental contexts. Not only *lin-41* mutants, but also another class of mutants has been identified in the screen and started to be characterized. The oocytes of the mutants belonging to this class prematurely enter an embryonic state, but, despite that, they are not able to fully acquire pluripotent features and to differentiate into somatic cells. Overall this thesis has been able to shed some light on the factors and, at least in part, the post-transcriptional mechanisms controlling pluripotency during the oocyte-to-embryo transition.

Introduction

1. Pluripotency and stem cells

1.1. Why studying stem cells

During eukaryotic sexual reproduction, the fusion of two highly specialized and haploid cells, the oocyte and the sperm (the gametes), gives rise to a single cell, the zygote. The zygote is a diploid cell which has the ability to give rise to any embryonic (and extra-embryonic for some species) cell of the three germ layers (endoderm, mesoderm and ectoderm), which will allow the proper development of the newly formed organism. To enable the maintenance and the propagation of the species, the great plasticity (named totipotency) possessed by the zygote has to be somehow retained in the cells that will originate the gametes, *i.e*., the germ cells. Understanding how a fully developed organism can arise from a single cell and how such a developmental information can be inherited and transmitted generation after generation constitute an intriguing challenge for biologists.

The "potency" of a cell is defined based on its ability to differentiate into a certain range of differentiated cell types: as already mentioned, toti-potency, characteristic of the zygote, describes cells which are able to originate both embryonic and extra-embryonic cells; pluri-potency is defined as the ability of a cell to give rise to any differentiated embryonic cell, whereas multi- and uni-potency restrict their ability to a few (multi-) or one (uni-) cell types.

In the last decades, scientists have developed more and more interest in the field of pluripotency, discovering that not only zygote and germ cells, but even in the adult organism stem cells can be found and they allow the physiological homeostasis of the organism as well as regenerative abilities (Pellettieri and Sànchez, 2007, for a review on the topic). *In vitro*, the use of cell culture allowed to indefinitely maintain cell pluripotency through the creation of cultured embryonic stem (ES) cells, which are able to self-renew without undergoing differentiation (Evans and Kaufman, 1981; Martin, 1981). The main questions scientists have been trying to unravel regard what factors and mechanisms underline this fundamental developmental process and how, in normal development, cell fate decision is made. Answering these basic biological questions wouldn't be a simple satisfaction of understanding the "secret of life", but it would be more practically useful for medical purposes: finding a clean and precise way to specifically and robustly drive a certain differentiation pathway, from undifferentiated stem cells, would provide an enormous contribution to regenerative medicine, allowing to bypass all the issues determined by heterologous transplantations (*e.g.*, tissue rejection, immunosuppression etc.).

1.2. Types of stem cells: an overview

The study of pluripotent stem cells started in the 1950s with teratocarcinomas, malignant germ cell tumors formed by embryonal carcinoma (EC) cells which can differentiate into somatic cells (Stevens and Little, 1954). Later on, in the 1970s, mouse EC cell lines could be propagated *in vitro* and were regarded as "*in vitro* caricatures of development" for their ability to mimic embryonic development in maintaining their undifferentiated state and being able to differentiate into any cell of the three embryonic germ layers, while restricting their developmental potential (Kahan and Ephrussi, 1970; Yu and Thompson, 2008).

A step further was provided by the discovery that the transfer of early mouse embryo into extrauterine sites could cause teratocarcinoma formation, suggesting that these embryonic cells could retain pluripotent abilities for the first few cell cleavages (Solter et al., 1970; Stevens, 1970). From this discovery on, scientists have been able to derive *in vitro* cell lines from ES cells (Evans and Kaufman, 1981; Martin, 1981) which, differently from EC cells in being karyotypically normal, could give rise to a variety of tissues in chimera animal models (organisms constituted by genetically different cells), including germ cells, and, therefore, allowing the introduction of desired modifications in the germline (Bradley et al., 1984). With a delay of a little less than 20 years, also karyotypically normal human ES cell lines were created and could retain their pluripotent abilities even after prolonged undifferentiated cloning, providing new prospectives for regenerative medicine (Thomson et al., 1998; Yu and Thompson, 2008).

Despite the fact that teratocarcinomas were known to be derived from primordial germ cells (PGCs), it took about thirty years to scientists to be able to create *in vitro* mouse embryonic germ (EG) cell lines from PGCs (Matsui et al., 1992; Resnick et al., 1992). EG cells are morphologically undistinguishable from ES cells, although they differ in some epigenetic aspects, such as genome-wide demethylation, no genomic imprinting and active X chromosomes (Labosky et al., 1994; Tada et al., 1997). Soon after the establishment of mouse EG cell lines, also human EG cells were created, but their proliferative potential seems to be limited in a time scale (Shamblott et al., 1998; Turnpenny et al., 2003; Yu and Thompson, 2008).

A great glimpse into the field of stem cells has to be attributed to the studies on nuclear reprogramming, a process through which the differentiated state of a somatic cell can be experimentally reversed to the one of another cell type (Gurdon and Melton, 2008). Two major experiments, which led

to the assignment of the Nobel Prize in 2012, contributed to the discovery that differentiated cells can be reprogrammed into pluripotent cells. The first was done by John B. Gurdon, who demonstrated that the nucleus of a somatic cell, transferred into an enucleated *Xenopus laevis* egg, could be reprogrammed during the so-called somatic cell nuclear transfer (SCNT) and give rise to a fully functional adult organism (Gurdon and Uehlinger, 1966). The second one was done by Shinya Yamanaka's group members who were able to create induced pluripotent stem (iPS) cells through viral transfection of four specific transcription factors (Oct3/4, Sox2, c-Myc and Klf4) in differentiated cells under specific cell culture condition (Takahashi and Yamanaka, 2006).

1.3. Pluripotency and germ cells

Germ cells clearly possess a great developmental potential which, in normal development, is demonstrated during the so-called oocyte-to-embryo transition (OET). It is during this transition that the transcriptionally quiescent oocyte undergoes a series of reprogramming events and gives rise to a pluripotent zygote/early embryo. These reprogramming events do not only occur in normal development, but also in disease, where mutant germ cells can escape their meiotic cell cycle and originate teratocarcinomas cells (Stevens and Little, 1954). Furthermore, *in vitro*, under specific culture condition, germ cells can give rise to EC and EG cell lines and the oocyte or egg cytoplasms possess the ability to reprogram somatic cells into iPS cells.

1.3.1. Cytoplasmic factors controlling pluripotency in germ cells

Post-transcriptional gene regulation is a fundamental process to finely regulate gene expression levels in time and space. Similarly to transcriptional regulation, which is based on the recognitions of key elements in the promoter sequence of a certain gene by specific transcription factors (TFs), post-transcriptional regulation relies on the presence of critical binding sites mostly present on the 5' and 3' untranslated regions (UTRs) of a certain transcript by specific RBPs or non-coding RNAs (ncRNAs), more generally defined as cytoplasmic factors. As it will become clearer in the following paragraphs, post-transcriptional gene regulation plays a key role in regulating the transcriptional quiescent steps during oocyte formation and maturation, as well as in the early steps of embryogenesis, preceding the restart of polymerase (Pol) II-dependent transcription. We can, therefore, postulate that during the OET it is not the transcriptional, but the post-transcriptional gene regulation which controls the onset of pluripotency.

For many years the role of RBPs in controlling gene regulation has been underestimated. For this reason, not as many efforts have been invested in identifying key players of pluripotency among RBPs as they have been for TFs. Luckily, after the discovery of ncRNAs and their roles in controlling gene expression, much more interest and resources have been provided to studying post-transcriptional gene regulation, and so RBPs, also in the context of regulation of pluripotency (Ye and Blelloch, 2014, for a review on the topic). As mentioned before, precocious onset of pluripotency in germ cells can be reported with the formation of specific germline tumors, called teratomas. Therefore, an easy way to identify RBPs which are somehow involved in the regulation of pluripotency in the germline is to analyze which factors can more frequently induce the formation of this kind of tumor when mutated. Although teratomas can originate from both female and male germlines, so far, in mammals, only factors which enhance the frequency of testicular teratomas have been identified. The first factor with an RNA recognition motif to be directly implicated in a heritable cause of tumorigenesis, *i.e*., teratoma, was the murine gene *Dnd1*, ortholog of the *dead end* (*dnd*) gene of zebrafish. PGCs presenting a precocious stop codon in the *Dnd1* gene get transformed into undifferentiated pluripotent embryonal carcinoma cells, which will differentiate after birth (Youngren et al., 2005). DND1 has been later shown to exert its function via counteracting the function of different miRNAs, through the binding of the miRNA target transcript, on a uridine-rich sequence. In this way, DND1 prohibits the certain miRNA to bind its target and protects it from the miRNA-dependent translational repression (Kedde et al., 2007). Soon after the description of the involvement of DND1 in the control of pluripotency in PGCs in mouse, another RBP was discovered, to play a key role in preventing teratoma formation in the germline a worm model (Ciosk et al., 2006). This protein, GLD-1, controls the correct progression into meiosis of germ nuclei: in fact, when absent, germ nuclei are able to enter meiosis, but they cannot maintain this state and properly differentiate into gametes (oocytes). They, then, re-enter mitosis and differentiate into somatic cells, forming a worm teratoma (Ciosk et al., 2006). GLD-1 has been later shown to act as a translational repressor, preventing teratoma formation through the negative control of different targets, among which maternally provided embryonic cell fate determinants (*e.g*., PAL-1/CDX) and specific cell cycle regulators (CYE-1/Cyclin E) (Biedermann et al., 2009). Although its presence has been shown to be quite variable, a certain correlation between the presence of LIN28 and formation of teratomas in humans could be recently demonstrated (Cao et al., 2011). LIN28 was already known to maintain pluripotency in ES cells, but in the work from Cao et al., 2011, it seems it can be considered a marker for testicular germ cell tumor, too. Finally, a study from last year has introduced DAZL, a germline-specific RBP, as a factor which is able to limit pluripotency and the following differentiation in murine PGCs. As well as GLD-1,

DAZL seems to act as a translational repressor, having among its target transcripts some of the core pluripotency factors (*i.e.*, *Sox2* and *Sall4*). Taking that into account, although DAZL absence might theoretically lead to teratoma formation, this is not the actual phenotypic readout one does observe, as, among its targets, we can also identify mRNAs coding for certain caspases, which will activate the apoptotic cascade in the *Dazl* mutant gonads (Chen et al., 2014). DAZL is a good example to understand how the study of RBPs as translational repressors might sometimes be tricky, as these proteins might regulate several pathways in parallel and the simple look at the final phenotype might not be enough to understand what different biological aspects they have an impact on.

2. The oocyte-to-embryo transition

The OET constitutes a physiological developmental context to understand how pluripotency is established and, at the same time, kept at a bay in germ cells, or more specifically in oocytes. To identify the factors and the pathways which play a key role in germ cell pluripotency, one needs to have a clear idea of what the different developmental processes and molecular mechanisms occur during the physiological transition from a terminally differentiated and meiotic cell, the oocyte, to the reprogrammed, pluripotent and mitotic early embryo. The OET can be sub-divided into different molecular activities, which can be summarized in the following ones:

- Oocyte maturation (and fertilization);
- Degradation of maternal factors;
- The embryonic genome activation (EGA).

It is important to stress out that it is during this transition that the shift from a maternal to an embryonic developmental control occurs. All these three events are specifically controlled by maternally provided factors and only after the EGA, therefore after the re-start of Pol II-dependent transcription, the embryo will be able to look after its own development with mRNAs and proteins coded by its own genome. Furthermore, although different molecular mechanisms have been shown to play a role in controlling OET, many aspects of this transition still have to be elucidated. Despite that, it already appears clear that the mechanisms involved in this process are fully post-transcriptional, as OET occurs in the absence of Pol II-dependent transcription, making cytoplasmic factors, such as RNA binding proteins (RBPs), and not the much more publicized nuclear factors (*e.g.*, TFs and chromatin remodelers) the key players in this developmental context.

2.1. Oocyte maturation

To be competent to get fertilized, an oocyte needs to reach its full maturation. The molecular mechanisms and developmental timings underlying oocyte maturation vary in several aspects in different organisms. Despite that, it is possible to identify conserved biological and molecular processes which are shared among sexually reproducing eukaryotes. A first similarity can be found in the main steps during meiotic maturation (Fig. 1):

- Primary cell cycle arrest in prophase of meiosis I (MI);
- Maturation;
- Secondary cell cycle arrest;
- Egg activation, ovulation and fertilization.

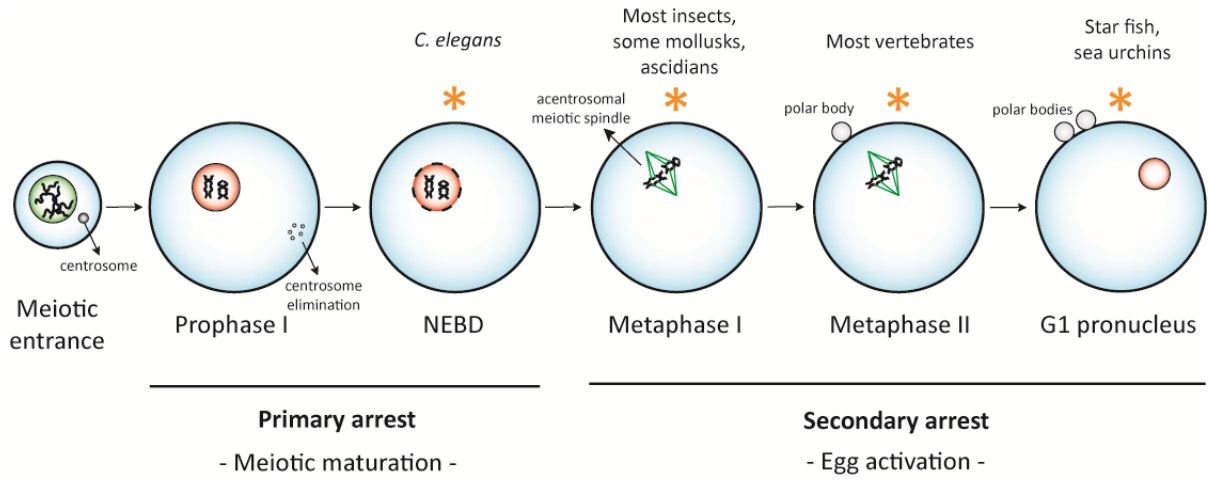

Figure 1. Meiosis in oocytes (adapted from Kim et al., 2013; Saganata, 1996; Von Stetina and Orr-Weaver, 2011): Schematic representation of the different with the main features a developing oocyte undergoes. Here and in the following figures: the outer blue circles represent the cytoplasms and the inner green (transcriptionally active) or red (transcriptionally inactive) ones represent the nuclei. Asterisks mark when fertilization occur in the species named above them.

After entering meiosis, but before reaching the end of prophase I, when the developing oocyte will undergo its first cell cycle arrest, two key events have to occur: the centrosome has to be eliminated to prevent abnormalities in the karyotype of the embryo, as a new and functional one will be paternally supplied by the sperm (Fig. 1) (Albertson et al., 1993; Schatten and Sun, 2011). The second event consists in the inhibition of Pol II-dependent transcription (Fig. 1), which will resume at species-specific time point during embryonic development (Tadros and Lipshitz, 2009). Therefore, before the transcriptional inhibition takes place, the oocyte cytoplasm has to contain all the mRNAs which will be

necessary not only to continue oocyte development, but also to start embryogenesis. Furthermore, it appears clear that in this scenario post-transcriptional gene regulation results the key mechanism through which the oocyte and, later on, the early embryo can control their development. It is not fully understood why this transcriptional pausing occurs, but the fact that this step is so highly conserved might suggest being essential in proper oocyte development.

Depending on the species, at the diplotene or diakinetic stage of prophase I, the oocyte will undergo its first cell cycle arrest, which constitutes the sole one for *C. elegans* (Von Stetina and Orr-Weaver, 2011). This pause can even last up to several years (*e.g*., in humans) and allows the oocyte to get ready for fertilization, undergoing its meiotic maturation: upon a specific trigger (*e.g*., luteinizing hormone in *H. sapiens* or the major sperm protein (MSP) in *C. elegans*), the oocyte will be subjected to a series of specific cytological changes, such as nuclear envelope break down (NEBD), rearrangements in the cortical cytoskeleton and meiotic spindle assembly (Kim et al., 2013). The factor regulating the entrance into meiotic maturation is the so-called maturation promoting factor (MPF), which was first discovered by Yoshio Matsui in his work on frogs (Masui and Markert, 1971) and it was, then, proved to be the master regulator in such developmental context in all the examined model organisms (Boxem et al., 1999; Von Stetina and Orr-Weaver, 2011). After its discovery, MPF was shown to be constituted by a protein complex composed by two factors: a catalytic subunit, Cdk1 (also known as Cdc2), and a regulatory one, cyclin B (Dunphy et al., 1988; Gautier et al., 1988; Lohka et al., 1988). Not only MPF, but also its direct regulators appear to be highly conserved, although the mechanisms and cascades which allow them to exert their functions exhibit some variations from organism to organism (Von Stetina and Orr-Weaver, 2011). Cdk1, already present during the first oocyte arrest, is maintained inactive by the kinases Wee or Myt1, which phosphorylate Cdk1 in two conserved residues, tyrosine 14 and 15 (Fig. 2) (Mueller et al., 1995). Such phosphorylations can be removed by the dual-specificity phosphatase, Cdc25, which gets activated upon the signal for resumption of meiosis (Kumagai and Dunphy, 1991): MPF is now active and it is able to start meiotic maturation (Fig. 2).

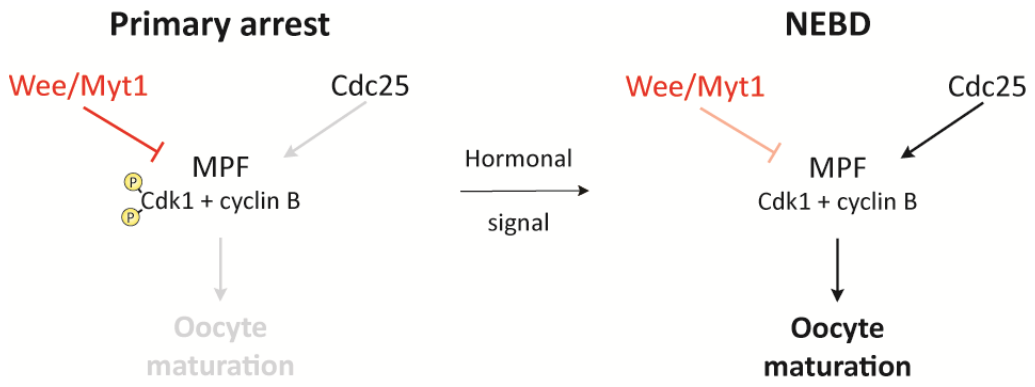

Figure 2. Regulation of MPF during the primary arrest and its release: Schematic description of MPF regulation by the two inhibitory kinases Wee and Myt1 and the phosphatase Cdc25 in directing the first meiotic arrest versus oocyte maturation.

After meiotic maturation, a second cell cycle arrest occurs in most animals and lasts until fertilization (Fig. 1). The specific time point this secondary arrest happens varies from species to species as well as ovulation, which normally occurs during this period. Nevertheless, it seems that high levels of active Cdk1/cyclin B are critical in controlling the maintenance of this phase (Oh et al., 2013). The completion of meiosis, indeed, requires the anaphase promoting complex/cyclosome (APC/C) activity, which targets cyclin B for degradation and allows the relive of the meiotic block (Horner and Wolfner, 2008; Nixon et al., 2002).

In most of the cases, egg activation (*i.e.*, completion of meiosis) occurs upon fertilization, but there is one example, represented by *Drosophila melanogaster*, in which egg activation is independent from fertilization and it is triggered by a mechanical stimulus when the egg passes through the oviduct (*i.e.*, ovulation; Mahowald et al., 1983).

2.2. Degradation of maternal factors

As previously mentioned, the egg gets loaded with a great amount of maternal mRNAs and proteins, which will allow virtually any aspect of the early embryonic development. After the formation of the zygote, maternally provided mRNAs and proteins get inhibited and degraded by maternally encoded products. Only after the EGA, the embryo will complete this clearance with the products coded by its own genome and continue its development.

Upon fertilization, the oocyte intracellular calcium concentration increases and initiates egg activation with a series of molecular events (Horner and Wolfner, 2008). Among them we can identify

the release of meiotic arrest, the generation of parental pronuclei, cytoskeletal rearrangements and the changes in maternal factor populations (Horner and Wolfner, 2008). Although the exact connection between calcium signaling and maternal factor destabilization remains unclear, studies in different model organisms have identified several post-transcriptional and post-translational pathways which coordinate this event. In *D. melanogaster*, for example, two different mechanisms have been suggested: the first one involves Pan gu (PNG), a Serine/Threonine kinase, already shown to be required for the onset of mitotic division in the embryo (Fenger et al., 2000; Shamanski and Orr-Weaver, 1991). In a first study, PNG has been shown to promote the translation of the RBP Smaug (SMG) (Tadros et al., 2007). SMG specifically binds SMG recognition elements (Smibert et al., 1996) present in maternal mRNAs and its binding allows the recruitment of the CCR4/POP2/NOT complex, which, with its activity as a deadenylase, removes the poly(A) tails from its targets and allows their destabilization, which constitutes the first step towards their degradation (Tadros et al., 2007; Semotok et al., 2005). In a more recent study, PNG has been proposed as the key regulator for the changes in the translatome which can be observed during *D. melanogaster* OET, both inhibiting or activating translation of a great number of mRNA, mostly allowing a balance of the protein levels against protein degradation (Kronja et al., 2014). The second postulated mechanism derives from computational analyses on the sequences of destabilized maternal transcripts (De Renzis et al., 2007). In this study, two *cis*-elements have been found to be enriched in the mRNA sequences of destabilized maternal transcripts: one resembles the PUF-family binding site and the other one the AU-rich *cis*-elements (AREs), but the potential roles of Pumilio and ARE-binding proteins in maternal mRNA destabilization haven't been fully proven in *D. melanogaster*. Despite that, in *X. laevis*, the ARE-mediated pathway does, indeed, work together with the embryonic deadenylation element binding protein to trigger the deadenylation of maternal transcripts upon fertilization (Paillard et al., 1998), although it has to be mentioned that, in this model organism, fertilization-induced deadenylation does not trigger mRNA decay until after the EGA (Audic et al., 1997; Duval et al., 1990; Voeltz and Steitz, 1998). In *C. elegans*, a recent study has quantified and analyzed transcriptome-wide, expression of mRNAs and thousands of proteins in oocytes, 1-cell and 2-cell stage (c.s.) embryos (Stoeckius et al., 2014). This work could show that shortly after fertilization thousands of mRNAs are eliminated and that this clearance is highly significantly dependent on the presence of a poly(C) motif in the 3' UTRs of these transcripts. Furthermore, additional data indicate that endogenous siRNAs, but not miRNA, promote mRNA clearance during OET. This last evidence is in contrast to what has been previously suggested for other model organisms, where miRNAs seem to be

mediators of the zygotic degradation pathway (Benoit et al., 2009; Bushati et al., 2008; Giraldez et al., 2006).

2.3. The embryonic genome activation

The EGA is another highly conserved process which occurs at species-specific time points in all eukaryotes and it implies the reactivation of Pol II-dependent transcription in the embryo, after its inhibition during oocyte formation. It is easy to understand that also this process is controlled by maternally provided factors present in the oocyte, which need to be somehow "triggered" to exert their functions at the specific time point the EGA is supposed to occur. The importance of the EGA relies on the fact that the newly provided factors allow embryogenesis to proceed, by directing the next steps of the developmental program: upon embryonic transcriptional inhibition, indeed, embryos of any studied model organism exhibit severe cytological and developmental defects (Lee et al., 2014), such as appearance of morphological defects for *C. elegans*, where the embryo does still manage to reach the 100 c.s. (Edgar et al., 1994), failure in cellularization for *D. melanogaster* (Edgar et al., 1986; Merrill et al., 1988) and in undergoing gastrulation for *X. leavis* and *Danio rerio* (Kane et al., 1996; Newport and Kirschner, 1982a) and block at the 2 c.s. for *Mus musculus* (Goddard and Pratt, 1983; Golbus et al., 1973; Warner and Versteegh, 1974). As previously mentioned, the EGA occurs in each species at specific and tightly regulated time points (or number of cell cleavages) after fertilization and they can go from 1 to 2 cell cycles in *M. musculus*, to 6 to 9 cycles in *X. leavis* and *D. rerio* (Lee et al., 2014). It has to be mentioned that from an absolute time prospective, the EGA happens in mammals much later than in other species, as the first cell division occurs after more than one day after fertilization, *e.g*., for *M. musculus* (Hamatani et al., 2004).

Different models, which describe how the onset of the EGA is controlled, have been proposed and are not mutually exclusive, suggesting that different layers of regulation may be needed to allow such a fine process. Among the most convincing models, it is possible to name the following ones:

- The nucleocytoplasmic (N/C) ratio;
- The maternal clock;
- The transcript abortion;
- The chromatin regulation.

The N/C ratio (*i.e*., the ratio of nuclear to cytoplasmic volume in a cell) model suggests that the transcriptional inhibition gets alleviated thank to the titration of repressive maternally provided factors by the exponentially increasing of nuclear material obtained after a certain amount of cell divisions (Fig. 3) (Newport and Kirschner, 1982a). The hypothesis that the N/C ratio may play a role in the EGA comes from the discovery that polyspermic *X. laevis* embryos are able to undergo the EGA two cell divisions before monospermic ones, suggesting that the onset of embryonic transcription in this model organism is not based on a critical amount of cell cleavages *per se*, but from an alleviation of repressive mechanism due to the amount of DNA present in a shared cytoplasm (Newport and Kirschner, 1982a; Newport and Kirschner, 1982b). A candidate transcriptional repressor in this model organism is the *X. laevis* homolog of DNA methyltransferase, which seems to be able to exert its role in this context independently of its catalytic activity: the catalytically-dead mutant, as well as the knock-out (K.O.) of such gene, shows, indeed, precocious EGA (Dunican et al., 2008; Stancheva and Meehan, 2000). Another example, which supports this model, comes from *D. rerio*: the failure of chromosomal segregation leads to the formation of polyploid cells (therefore with higher N/C ratio), which show premature transcriptional initiation (Dekens et al., 2003). In *D. melanogaster*, the scenario seems to be more complicated, where clearly more than one model may describe the onset of embryonic transcription. On the first place, different lines of evidence suggest that only a subset of genes are actually affected by the N/C ratio (Edgar et al., 1986; Lu et al., 2009; Yasuda et al., 1991). Furthermore, it has been recently proposed that the N/C ratio can affect the EGA only indirectly, through the regulation of the cell cycle (Lee et al., 2014): Tween, the Cdc25 homolog, has been shown to affect the cell cycle pause, observed before cellularization, and its degradation, probably occurring in a N/C ratio-dependent manner, seems to stabilize the phosphorylated and inactive form of Cdk1 and, therefore, preventing the entry into mitosis and allowing the EGA to occur (Di Talia et al., 2013; Farrell and O'Farrell, 2013).

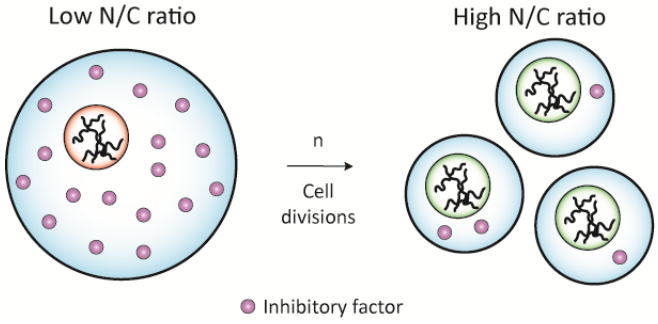

Figure 3. The nucleocytoplasmic ratio model: Schematic representation of the N/C ratio model, showing how a certain inhibitory factor (purple dot) gets titrated with the increasing amount of cell division and allows transcription to start (from red to green schematic nuclei).

The maternal clock model proposes the existence of a cell cycle-independent "clock", which starts counting the time from the occurrence of egg activation or fertilization and can determine the timing the EGA should start at, either by activating/producing the transcriptional machinery or by de-repressing embryonic transcription (Fig. 4) (Howe et al., 1995; Tadros and Lipshitz, 2009). Post-transcriptional and post-translational mechanisms have been proved to be essential in regulating the EGA and to be activated upon the start of the maternal clock. In two different model organisms, *X. laevis* and *M. musculus*, the EGA appears to be regulated by degradation or translation, respectively, of cell cycle regulators (Hamatani et al., 2004; Hara et al., 2005; Howe et al., 1995; Howe and Newport, 1996): the changes in the levels of such factors seem to be N/C ratio-independent, suggesting that a specific trigger, such as fertilization, induce them to occur. As previously described, in *D. melanogaster* SMG gets translated and destabilize the majority of maternal transcripts upon egg activation (Tadros et al., 2007). Furthermore, SMG has been shown to be required for the high-level transcription during the EGA (Benoit et al., 2009). Finally, post-transcriptional events seem to be key regulators of the EGA in *C. elegans*: the MBK-2-dependent phosphorylation of the two zinc finger proteins OMA-1 and OMA-2, occurring upon fertilization, allows them to bind and sequestrate in the cytoplasm TAF-4, one of the crucial components for the assembly of the transcription factor-II D and the RNA Pol II pre-initiation complex (Guven-Ozkan et al., 2008). With a non-fully clear mechanism, this phosphorylation on the two OMA proteins also marks them for degradation, which occurs just before the 4 c.s. of the embryo: at this point, TAF-4 is free to reach the nucleus and embryonic transcription can start (Guven-Ozkan et al., 2008).

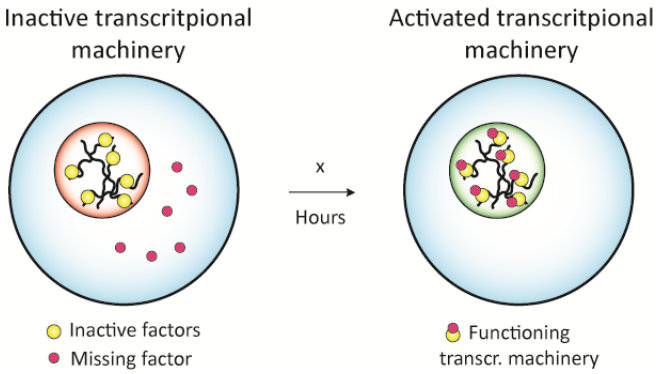

Figure 4. The maternal clock model: Schematic representation of one example on how the maternal clock model can act: inactive factors (*e.g.*, the *C. elegans* pre-initiation complex – yellow dots) are inactive, but already sitting on the chromatin. A missing factor (*e.g.*, the *C. elegans* TAF-4 – purple dots) will enter the nucleus only after a certain time fertilization occurred, bind the pre-initiation complex and allow transcription to start (from red to green schematic nuclei).

The transcription abortion model postulates that embryonic mRNAs start to get transcribed before the actual EGA, but their transcription is incomplete due to the rapid DNA replication during the early cleavage cycles (Fig. 5) (Edgar and Schubiger, 1986; Kimelman et al., 1987; Tadros and Lipshitz, 2009). Blocking the cell cycle in *X. laevis* and *D. melanogaster* leads, indeed, to a premature EGA (Edgar and Schubiger, 1986; Kimelman et al., 1987). The facts that, in *D. melanogaster*, the majority of the early embryonic transcripts are intron-less and code for small peptides (De Renzis et al., 2007) and relatively large genes undergo, instead, abortive transcription (Shermoen and O'Farrell, 1991) fully confirm the validity of the proposed model.

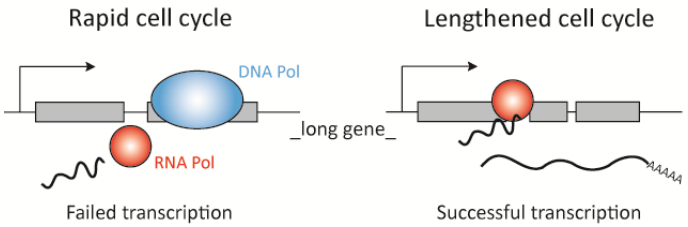

Figure 5. The transcript abortion model (adapted from Lee et al., 2014): Schematic representation of a failed (left) or a successful transcription (right) of a long gene (grey boxes are the exons, whereas black lines are the introns and the UTRs) during a rapid (left) or a lengthened cell cycle (right): the failing in RNA Pol II-dependent transcription of the first case is due to the overcome of the DNA Pol on the DNA sequences.

Finally, the chromatin regulation model hypothesizes that the early embryonic chromatin is not immediately competent for transcriptional activation, as it derives from the transcriptionally quiescent oocyte and sperm. Therefore, only after chromatin modifications have occurred, it is possible to assist to the EGA (Fig. 6) (Tadros and Lipshitz, 2009). This model would suggest that the transcriptional machinery is already ready to exert its job, but the presence of repressive markers at the chromatin level prevents them to start embryonic transcription. Studies in different model organisms, where a plasmid containing an exogenous gene was injected in the early embryo before the EGA, could show that it could get actually transcribed (Newport and Kirschner, 1982b; Wiekowski et al., 1993). Thus, these results could confirm the hypothesis that the transcriptional machinery of the early embryo is, indeed, competent to start transcription if a permissive chromatin landscape is present. The general mechanisms used by embryonic cells to modify the silenced chromatin and allow the transcription machinery to start the EGA are:

- Histone exchanges: gamete-specific variants are replaced by somatic versions (Faast et al., 2001; Fu et al., 2003; Lee et al., 2014; Pérez-Montero et al., 2013; Smith et al., 1988; Tanaka et al., 2001; Whittle et al., 2008);

- Histone modifications: inactive histone tail marks (*i.e.*, H3K9 and H3K27 methylation) are removed and active ones are established (*i.e.*, H3K4 and H3K36 methylation, H4 acetylation) (Adenot et al., 1997; Akkers et al., 2009; Bultman et al., 2006; Lindeman et al., 2011; Schuettengruber et al., 2009; Sun et al., 2007; van der Heijden et al., 2006; Vastenhouw et al., 2010).

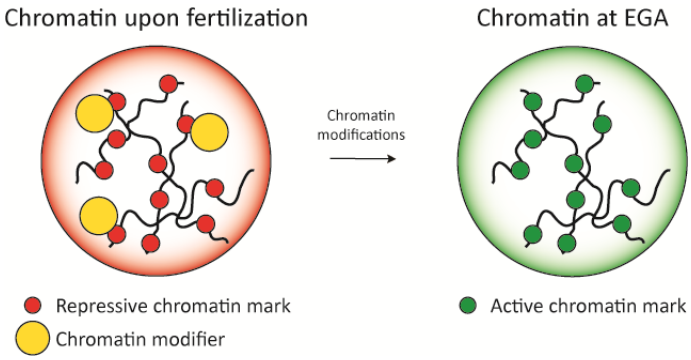

Figure 6. The chromatin regulation model: Schematic representation of an embryonic nucleus which is transcriptionally incompetent (red) upon fertilization (left) and can become transcriptionally active (green) and undergo EGA only when chromatin modifiers (yellow dots) will convert the repressive chromatin marks (red dots) into active ones (green dots).

To make the scenario even more complicated, it is worth mentioning that, in addition to the mechanisms described by these four models, an additional layer of regulation is provided by TFs and their co-factors, which specifically mediate the EGA. Not much is known about which factors play this role and how. Anyway, a couple of examples are provided by studies in *D. melanogaster* and *D. rerio*: in the first one, early embryonic genes possess heptamer DNA motives, known as TAGteams, in their promoter sequences, which are bound by the TF Zelda (De Renzis et al., 2007; ten Bosch et al., 2006); if Zelda is removed, among the different defects which can be observed, 120 early embryonic genes fail to get activated (Liang et al., 2008). In *D. rerio*, instead, early embryonic genes are enriched in their promoter sequences in binding sites for the homologs of the commonly known "pluripotency-inducing factors" (Lee et al., 2014), Nanog, Pou5F3 (homolog of the mammalian Oct4) and Sox19b (ortholog of Sox2) (Lee et al., 2013; Leichsenring et al., 2013), providing a conceptual bridge between the *ex vivo* reprogramming in iPS cells and the *in vivo* reprogramming occurring during the OET.

3. *Caenorhabditis elegans* as a model system to study germ cells

C. elegans is a one millimeter-long, free-living soil nematode (Fig. 7), which can be found in different geographical regions and primarily feed bacteria. It is a great and handful model organism to study germ cell development and the OET for many different reasons: its short life cycle (roughly three days under optimal conditions) and easy cultivation, its fully sequenced genome and invariant cell lineage, its transparency which allows to follow germline and embryonic development in intact animals and, most of all, the great abundance of germ cells and large number of progeny per worm (up to 300) (Brenner et al., 1974; *C. elega*ns Sequencing Consortium, 1998; Sulston and Horvitz, 1977).

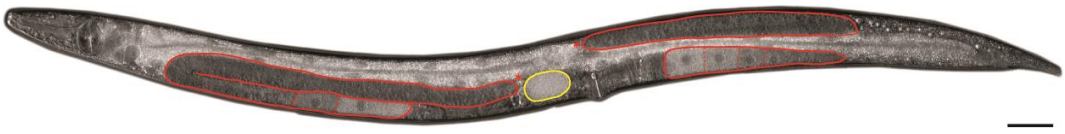

Figure 7. ***C. elegans***: A live differential interference contrast (DIC) picture of a young adult *C. elegans*. The two U-shaped gonads are highlighted in red and the first formed zygote in yellow. Scale bar: 50 µm.

3.1. *C. elegans* development

During its three-day long life cycle, the worm undergoes an embryonic stage, four larval stages (from L1 to L4) and adulthood. The embryonic development can be subdivided into two main stages:

- Proliferation, during which most of the embryonic cells are created, but are maintained in an almost undifferentiated state. This stage is further divided into two phases:
    - From the formation of the zygote until when embryonic founder cells are generated;
    - From the onset of gastrulation, at around the 30 c.s., to the beginning of organogenesis;
- Organogenesis/morphogenesis, during which terminal cell differentiation occurs without many additional cell divisions (Sulston et al., 1983).

From the first larval stage (L1) until adulthood, the worm grows in size, apoptosis of certain somatic cells occurs (n = 131), while the others (n = 959) reach their final differentiation and the organs get fully developed. The end of each larval stage is marked by a molt, during which the worm enters a period of lethargus while it sheds its collagenous cuticle and a new one gets synthetized by the underlying epithelium. Once the worm reaches adulthood, it becomes mature for reproduction and the three-day life cycle arrives at its end once it lays the first egg; the egg-laying period lasts about 4 days and, after that, adults can further live another additional 10-15 days (Byerly et al., 1976). *C. elegans* has

two genders: the self-reproductive hermaphrodite (XX) and the male (XO), which karyotypically differs from the hermaphrodite for the lack of one of the two X chromosomes (Sulston et al., 1980). Males arise infrequently (0.1%) due to non-disjunction of the X chromosome in the hermaphrodite germline; higher rates of male incidence can be observed after exposing worms to stress (*e.g.*, heat-shock).

3.2. Development of the reproductive system

The hermaphrodite reproductive system is constituted by a somatic gonad, the germline and the egg-laying apparatus (Kimble and Hirsh, 1979). Two bilaterally symmetric U-shaped tubular gonads are connected to a central uterus through a spermatheca. In the distal syncytial part of the gonad, a pool of proliferating nuclei is placed, whose function is to provide a continuous amount of nuclei to create new gametes (Ellis and Kimble, 1994). This pool of nuclei undergoes mitosis thank to a single cell, called the distal tip cell (DTC), which surrounds the distal gonad, constituting the niche of such compartment, and provides a GLP-1/Notch-dependent signal allowing the nuclei to proliferate (Crittenden et al., 1994). As these nuclei proceed more proximally, they enter the prophase of meiosis I and get cellularized in the loop zone. These cells are now developing oocytes, which, in the proximal gonad, form a row until the very proximal end and are transcriptionally inactive: here, it is possible to find the so-called -1 oocyte, which has reached its mature form, its nucleus is arrested in diakinesis of meiosis I and it is ready to get fertilized by sperm after being pushed inside the spermatheca (ovulation). Once the zygote is formed, it is moved into the uterus, where its first cell cleavages occur. At the 4 c.s. embryonic transcription starts in the somatic blastomeres (Fig. 8). After a few cell cleavages, the embryo gets laid outside from the vulva.

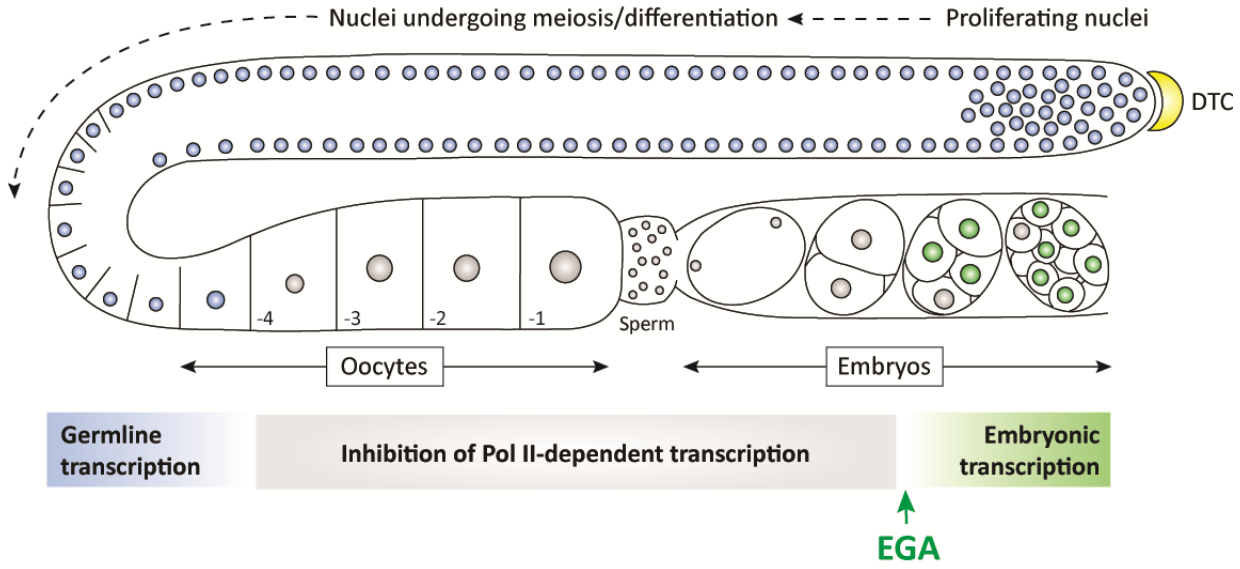

Figure 8. Transcriptional activity in the *C. elegans* germline and early embryos: A cartoon representing how transcriptionally active nuclei of the *C. elegans* germline become transcriptionally inactive as soon as they get cellularized and form oocytes. Upon fertilization, at the 4 c.s. of the embryo, Pol II-dependent transcription gets re-activated in the somatic blastomeres.

3.2.1. During embryogenesis

Differently from other organisms, germ cells are immediately established during embryonic development and set apart from the somatic ones. After the formation of the zygote, also referred ad P0, four asymmetric cell divisions will give rise to the primordial germ cell, P4. P4 gets internalized during gastrulation and will divide again only at around the 100 c.s., giving rise to the germline precursor cells Z2 and Z3. When the worm hatches the two cells Z2 and Z3 constitute the primordial germ cells (PGCs), the sole cells which will originate the germline, whereas the mesodermal somatic gonadal founders are Z1 and Z4, which migrate through an unknown mechanism, Z2 and Z3-independent, towards the two PGCs, and will give rise to all the somatic components of the reproductive system (Hubbard and Greenstein, 2000; Sulston et al., 1983).

3.2.2. During the larval stages

During the four larval stages, the hermaphrodite reproductive system development occurs as follow (Kimble and Hirsh, 1979):

- **L1 stage**: Z2 and Z3 start their divisions, which will continuously occur during all larval stages; in the second half of the stage, Z1 and Z4 produce 12 cells, among which we can also identify the vulval precursors.
- **L2 stage**: Z2 and Z3 daughters keep dividing, quadruplicating in their number. No further cell division occurs for the daughter cells of Z1 and Z4.
- **L3 stage**: at the L2/L3 molt, the 12 daughter cells of Z1 and Z4 start rearranging their localization to organize the future gonad, *e.g*., DTCs get positioned at the end of each gonad and start coordinating their elongation. Germ cells further proliferate thank to the signal received by the DTCs. Gonad arms start turning dorsally towards the mid-L3. During the whole stage, the somatic gonad precursors give rise to a total of 143 cells, which form gonadal sheet cells, spermathecae and uterus.
- **L4 stage**: the distal gonad arms finish their elongation and meiosis starts at the L3/L4 molt. Sperm starts its differentiation in the proximal gonad arms and it will be finally located inside the spermatheca. Vulval cells get fully differentiated. Gonadogenesis gets completed at this stage.

3.2.3. During adulthood

During the L4/adult molt, meiotic cells switch their developmental program and start to differentiate into oocytes (Ellis and Kimble, 1994). The amount of progeny per worm is sperm-dependent: if no males are around, the hermaphrodite will be able to fertilize a certain amount of oocytes, corresponding to the number of sperm located in the two spermathecae.

3.3. The embryonic genome activation as a marker for the onset of pluripotency

During normal development and, more specifically, during the OET, upon a specific trigger, *i.e*., fertilization, a terminally differentiated cell, the oocyte, undergoes a series of reprogramming events which will allow the erase of the previous developmental program and place the new informations for the establishment of pluripotency. The blastomeres of the early embryo, as previously described, possess pluripotent features, as they are able to give rise, both *in vivo* and *in vitro*, to any differentiated cell of the three germ layers. One of the very early steps during embryogenesis is the EGA, which has been also suggested to be the time point at which the major reprogramming events occur (Akkers et al., 2009; Vastenhouw et al., 2010). These two evidences can make one hypothesize that the EGA could be

used as a marker for the onset of pluripotency. Furthermore, in a previous study on the *C. elegans gld-1* mutant, germ cells, which form a teratoma and, therefore, precociously enter a pluripotent state, transcribe early embryonic mRNAs before getting fully differentiated (Biedermann et al., 2009). This study provides another evidence that, indeed, the EGA can be considered as a marker for the onset of pluripotency.

With the will to identifying new germline regulators which can prevent the precocious onset of pluripotency in germ cells, we decided to conduct a genetic screen, using the mutagen ethyl methanesulfonate (EMS), on a worm strain carrying a GFP reporter for the EGA. The EGA-GFP, which, in the wild-type situation, is specifically expressed in embryos, was used as a readout to assess pluripotency, when mis-expressed in mutant germlines.

EMS is a typical mutagen to create mutants in *C. elegans*: it induces point mutations scattered throughout the genomes by nucleotide substitution. Chemically, the ethyl group of EMS reacts with guanines and forms an abnormal base O-6-ethylguanine, which is frequently recognized as an adenine by the DNA replication machinery. Therefore, upon DNA replication, instead of the cytosine, originally opposed to the guanine which has been modified, a thymine is placed. With further rounds of replication, the original base pair G:C undergoes a genetic transition to an A:T (Merck, 1989).

The screen resulted in the identification of two distinct phenotypic classes of mutants which will be described in more details in the results section:

- **Class I**: sterile worms with an overall normal gonad which mis-express the EGA reporter in developing oocytes which present minor cytological defects. Two mutants have been found belonging to this class: a hypomorfic allele of the *gld-1* gene (Daubner et al., 2014) and a mutant in the *drh-3* gene (Fassnacht et al., in preparation).
- **Class II**: sterile worms exhibiting a somatic (Dpy) phenotype, with major defects in the proximal gonad arm, where cells mis-express the EGA reporter. Two mutants with two independent point mutations which give rise to precocious STOP codons on the same gene, *lin-41*, were found to belong to this class (attached manuscript, Tocchini et al., 2014).

4. TRIM-NHL proteins and their roles in development and disease

4.1. Domain structure of TRIM-NHL proteins

LIN-41, also known as TRIM71, belongs to one of the nine subfamilies of TRIM proteins, namely TRIM-NHL (C-VII). Their classification is based on the presence of different domains located at the C-terminus of the TRIM domain (Short and Cox, 2006), which is, in this case, the so-called NCL-1/HT2A/LIN-41 (NHL) repeat domain, named after the first proteins where it has been identified. The TRIpartite Motif (TRIM), on its turn, is constituted by three main domains: a RING (Really Interesting New Gene) finger, one or, more commonly, two B-Box-type zinc fingers (BB1 and BB2) and a Coiled-Coil (CC) (Fig. 9). The TRIM domain is always located towards the N-terminus of the protein and the way how its domains are spatially ordered is highly conserved and so it seems to be the spacing between them (Reymond et al., 2001), suggesting that they co-evolved, probably to optimize a specific function which can only be exerted by them as a group.

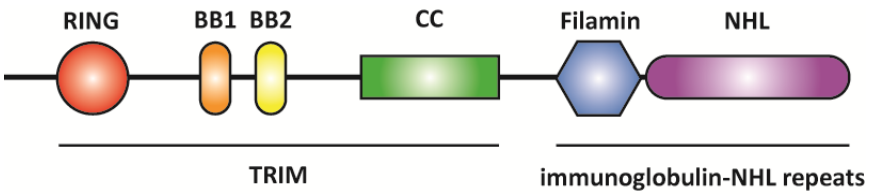

Figure 9. General domain structure of TRIM-NHL proteins: Highlight of the domains constituting the TRIM (RING, BB1/2 and CC) and the immunoglobulin-NHL repeats (Filamin and NHL) domains. The domains are aligned from the N- (left) to the C-terminus (right) as they normally occur in all the TRIM-NHL proteins.

4.1.1. The RING domain

The motif of the RING domain is defined by a regular series of cysteine (C) and histidine (H) residues which coordinate two zinc atoms in a "cross-brace" fashion, where Cs in positions 1, 2, 5, 6 bind the first atom and Cs and H in position 3, 4, 7, 8 bind the second one (Fig. 10; Barlow et al., 1994; Borden et al., 1995; Freemont, 1993). The eight conserved residues are located in the core of the domain, whose positions are guaranteed by the binding to the zinc atoms and they are essential in maintaining the proper ternary structure (Barlow et al., 1994; Borden et al., 1995). The RING domain has been shown to act as an E3 ubiquitin ligase. As an E3, it directly interacts with a specific E2 conjugating enzyme, which receives the ubiquitin signaling peptide from an E1 ubiquitin activating enzyme. The substrate of the reaction is specifically recognized and bound by other regions of the TRIM-NHL protein and gets mono- or poly-ubiquitinated on one or more lysine residues by the E2, brought to its proximity

by the RING domain. Notably, not all the RING domains can act as E3s and it has been recently demonstrated that to possess an E3 activity, a RING domain needs to possess a proline residue immediately after the C in position 7 (Budhidarmo et al., 2012). Ubiquitination is a post-translational modification which has been originally regarded as a mean to control protein levels through the proteasome system. More recently, other functions have been shown to exist for mono-ubiquitination: *e.g.*, regulation of protein activity and subcellular localization.

4.1.2. The two B-Boxes

The two BBs in the TRIM domain are also zinc binding motives and they come in two different versions (type I and type II), presenting similar, although distinct, consensus sequences (Fig. 10; Borden et al., 1993; Massiah et al., 2007; Reymond et al., 2001). The BB1 structure resembles the fold of RING, ZZ and U-box domains of E3 and E4 ubiquitin enzymes, suggesting that this domain may, in principle, either act as an E3 *per se* or enhance the RING domain activity as an E3 (Micale et al., 2012). Similarly to the RING domain, BB2 also coordinates its two zinc atoms in a "cross-brace" fashion (Micale et al., 2012). Nor clear or specific function has been demonstrated for the two types of BBs so far, although it has been proposed that, together with the CC domain, they provide the binding site for a specific substrate which will get ubiquitinated through the RING domain.

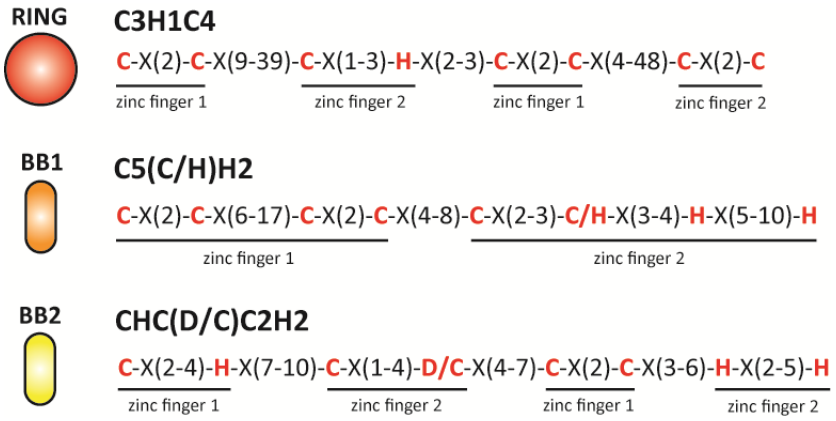

Figure 10. Domain consensi of RING, BB1 and BB2: Amminoacid consensus sequences of the three zinc-finger domains present in the TRIM domain: RING, BB1 and BB2. "X" stands for any amminoacid.

4.1.3. The coiled-coil domain

The CC is constituted by a region of roughly 100 amminoacids, whose primary structure is not conserved within the family. Despite that, the secondary structure usually presents a partition into two or three coiled-coil motives, mainly constituted by α-helixes which form a "rope-like" structure, stabilized by hydrophobic interactions and often mediated by leucine residues (Lupas, 1996; Micale et al., 2012). The CC domain allows the formation of homo- or hetero-dimers, promotes the formation of protein complexes (*e.g.*, recruiting the substrate for ubiquitination) and can help to define certain subcellular compartments (Reymond et al., 2001).

4.1.4. The immunoglobulin-NHL repeats domain

The filamin domain is often associated to the NHL repeats at the C-terminus of TRIM-NHL proteins (Meroni and Diez-Roux, 2005), suggesting a similar co-evolution already proposed for the domains of the TRIM motif. Its structure consists of a classic immunoglobulin-like domain, constituted by seven β-strands arranged in two antiparallel β-sheets (Bork et al., 1994), whose function, in the context of TRIM-NHL proteins, has been recently suggested to be connected to post-transcriptional gene regulation. More specifically, the filamin domain, together with the CC, is supposed to bind the proteins which would allow the translational inhibition of a certain transcript, bound, on its turn, by the NHL repeats (Loedige et al., 2013). The NHL domain is constituted by five or six repeats, of roughly forty residues each, and folds into a β-propeller structure, forming a disc: one of the two surfaces results to be highly positively charged and it has been recently proved to be the site interacting with mRNAs (Edwards et al., 2003; Loedige et al., 2014; Slack and Ruvkun, 1998). Therefore, NHL repeats appear to be involved in post-transcriptional gene regulation, although they have also been implicated in a more general role in mediating protein-protein interaction (Liu et al., 2014; Raheja et al., 2014).

4.2. TRIM-NHL proteins and their functions

TRIM-NHL proteins are emerging as key regulators in promoting differentiation and inhibiting cell growth and proliferation in stem and progenitor cells (Betschinger et al., 2006; Neumüller et al., 2008; Schwamborn et al., 2009). As it appears clear from the complex domain structure, these proteins seem to be definitely versatile in their functions and it has been suggested that different molecular mechanisms may be utilized by the same protein depending on the biological and physiological context

they are expressed. The proteins belonging to this class are not many and can be listed in a phylogenetic tree (Fig. 11).

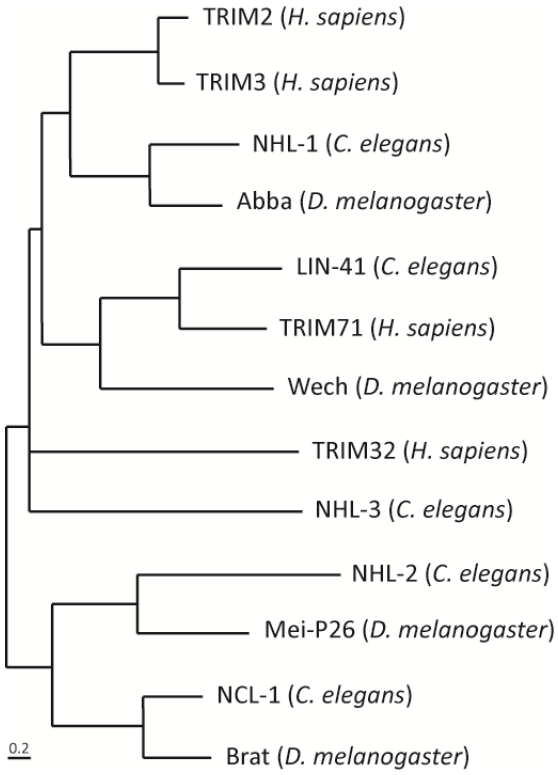

Figure 11. Phylogenetic tree of TRIM-NHL proteins (adapted from Hyenne et al., 2008): Phylogenetic tree showing the main proteins with their corresponding species in brackets belonging to the TRIM-NHL family. A scale bar for the relative evolutionary distance between the proteins is provided.

4.2.1. Brat

Brat is probably the most studied TRIM-NHL protein in developmental biology. The name *brat* derives from the phenotype (BRAin Tumor) observed in *D. melanogaster* larvae when such gene is mutated (Arama et al., 2000). In normal development, Brat controls the cell fate decision (differentiation versus self-renewing) of the daughter cells of larval neuroblasts, stem cell-like precursors of the adult brain. Specifically, Brat, together with the TF Prospero, is segregated into just one daughter cell (the so-called intermediate progenitor cell) and permits its differentiation, whereas the other cell, lacking these factors, will continue its self-renewal (Betschinger et al., 2006). Brat can be considered an atypical TRIM-NHL protein, as it lacks the RING domain. For this reason, it was originally regarded as a post-transcriptional regulator, whose main functional domain was the NHL. However, very recently, a novel mechanism sees the Brat BBs as key elements involved in the specification of the intermediate

progenitor cells. Such mechanism is suggested to be independent from asymmetric protein segregation, which appears, instead, to be mediated by CC and NHL repeats. Specifically, the BBs would be involved in repressing the β-catenin/Armadillo activity in the progenitor cells, preventing, in this way, their self-renewal (Komori et al., 2014). On the other hand, there are a bunch of evidences which suggests the NHL repeats to be, instead, the main player in preventing tumor formation. The first one came from a study where different point mutations in the NHL repeats were analyzed and could phenocopy the null allele (Arama et al., 2000). A further insight on Brat as a direct post-transcriptional regulator was provided by a four-hybrid interaction assay, where Brat was shown to be recruited to *hunchback* mRNA through the interaction with Nanos and Pumilio (Sonoda and Wharton, 2001). Furthermore, the authors could demonstrate that the interaction Brat-*hunchback* was NHL-dependent. The molecular mechanism through which the NHL repeats of Brat can bind a transcript through the help of Pumilio was described, in a not convincing way, a couple of years later (Edwards et al., 2003). Only recently, it has been conclusively proven that Brat directly binds *hunchback* mRNA and that the presumptive interaction with Pumilio was simply due to the proximity of Pumilio binding sites to the location where the Brat NHL repeats bind the target transcript (Loedige et al., 2014). A first clearer insight concerning which pathway(s) are affected by Brat's activity was provided by two studies not only on Brat, but also on its *C. elegans* ortholog, NCL-1. The authors suggested that the tumor phenotype in *D. melanogaster* was due to the lack of negative regulation of cell growth and ribosomal RNA synthesis normally provided by Brat in the wild-type situation (Frank et al., 2002). Such functions seem to be conserved as, in *C. elegans*, the lack of NCL-1 shows similar increasement in rRNAs and nucleoli size. Interestingly, these phenotypes can be rescue by the ectopic expression of Brat in worms (Frank et al., 1998; Frank et al., 2002). Furthermore, these data were confirmed and expanded by a transcriptome profile analysis which could provide a list of more than 300 genes, which were changing their expression levels in *brat* mutant. A gene ontology (GO) term analysis could show an enrichment in genes involved in metabolic and cell cycle activity, basal transcription machinery (and more generally transcriptional control) and ribosomal synthesis (Loop et al., 2004). Recently, Brat has been shown to function in other developmental contexts in *D. melanogaster*: it acts as a differentiation factor and inhibitor of cell renewal also in differentiating cytoblasts (cells derived from ovarian germline stem cells (GSCs)) (Harris et al., 2011) and it regulates neuromuscular synaptic growth in neuromuscular junction synapses (Shi et al., 2013). Interestingly, in both cases, Brat has been shown to act through its NHL repeats as a translational repressor of *mad*, which codes for the signal transduction effector of the bone morphogenetic protein (BMP) signaling pathway, therefore inhibiting cell renewal.

4.2.2. Mei-P26

Mei-P26, a *D. melanogaster* gene, was first discovered and characterized in the context of meiotic exchange and germline differentiation (Page et al., 2000). It appeared immediately clear that different roles could be attributed to this eclectic protein, depending on developmental time and context Mei-P26 is acting. The role of this protein in germline differentiation was strengthened years later, when it was shown that Mei-P26 could restrict growth and proliferation in the ovarian cell lineage, from the 16-cell cysts when it starts to be significantly present in germ cells: in its absence, such cells start to proliferate and form an ovarian tumor. Similarly to Brat, also Mei-P26 controls the differentiation of the *D. melanogaster* neuroblasts and nucleolar size, suggesting that a common mechanism could be shared among the two TRIM-NHL proteins. Such mechanism seems to involve the miRNA pathway as both proteins are able to bind Argonaute-1 and Mei-P26 can inhibit it (Neumüller et al., 2008). Controversially, another role, opposite to the one which has just been described, has been proposed for Mei-P26, showing its ability in maintaining the undifferentiated and proliferating state of GSCs by enhancing the translational inhibition provided by the miRNA pathway on certain transcripts. The absence of Mei-P26 would enable the precocious translation of Brat which, as previously described, would inhibit, on its turn, the BMP pathway, which is the final effector responsible for GSC proliferation (Li et al., 2012). Such controversy has been, at least in part, clarified by the hypothesis that Mei-P26 has two different functions in the germline depending on the compartment one takes into account: in GSCs, it maintains their proliferating state by enhancing miRNA-dependent silencing and promotes BMP signaling by repressing Brat (Harris et al., 2011; Li et al., 2012), but when the cells move away from the cap niche, Bag of marbles (Bag) gets expressed and allows the switch of Mei-P26 function. In these early differentiating cells, Mei-P26 promotes germ cell differentiation by negatively regulating miRNA pathway and probably changing its mRNA targets (*e.g.*, *nanos* transcript), by switching its interacting partners which are, in this context, Bam, Beningn gonial cell neoplasm (Bgcn) and Sex lethal (Sxl) (Li et al., 2013). Quite interestingly, the same factors (Bam and Bgcn) supposed to interact with Mei-P26 in the early differentiating cells in the female ovary have been shown, in the male gonad, to physically interact with another factor, Tumor testis (Tut), and translationally inhibit *mei-P26* and other transcripts, allowing, in this way, the proper control of proliferation versus differentiation (Chen et al., 2014). Eventually, Mei-P26 function has been analyzed not only in germ cells, but also in at least two other tissues: the main effector of Mei-P26, and also of Brat, seems to be dMyc, which is regulated by the first one at the protein level and at by the latter at the translational level in epithelial cells, but not only (Ferreira et al., 2014). Furthermore, in the nervous system Mei-P26 plays a major role in regulating

seizure susceptibility, probably having an impact on synaptic development (Glasscock et al., 2005). Despite the fact that the authors propose a role of Mei-P26 as an E3 ubiquitin ligase, the mutant they identified exhibits a missense mutation in the NHL repeats, suggesting a more probable role in post-transcriptional gene regulation.

4.2.3. TRIM2 and TRIM3

TRIM2, also known as NARF (Neural Activity-related RING Finger protein), and TRIM3, or BERP (Brain Expressed Ring finger Protein) are mammalian proteins closely related to each other, which are predominantly expressed in the brain where they exert their main functions in neuronal development and disease.

TRIM2 was first discovered as a factor involved in neuronal plasticity in *M. musculus*, where it was characterized as a candidate partner of myosin V, whose interaction was shown to be mediated by the NHL repeats (Ohkawa et al., 2001). TRIM2 seems to play a broader role in the brain by preventing neurodegeneration, acting as a UbcH5a-dependent E3 ubiquitin ligase and regulating the levels of the neurofilament light subunit (NF-L) in mice. If NF-L degradation gets impaired, it accumulates and determines axonopathy, followed by progressive neurodegeneration (Balastik et al., 2008). The very same conclusions are drawn by a study on humans, where a patient with a childhood onset of axonal neuropathy was discovered to have the gene coding for TRIM2 mutated (Ylikallio et al., 2013). A further role in the development of the nervous system has been shown using cultured mouse hippocampal neurons: the removal of TRIM2 via RNAi determines loss of neuronal polarity and no axon can be made. On the contrary, when TRIM2 is overexpressed, multiple axons are created, suggesting a key role of this factor in neuronal polarization and axon outgrowth during normal development, functions mediated by the regulation of NF-L levels as previously described (Khazaei et al., 2011). TRIM2 doesn't seem to be involved only in normal development, but also in preventing alterations of the physiological state when perturbations occur. For example, it has been shown to be involved in mediating the mitogen activated protein kinase (MAPK)-dependent ubiquitination of the cell death-promoting factor Bcl-2-interacting mediator of cell death (Bim) to allow the tolerance in episodes of brief ischemia (Thompson et al., 2011). A change in its levels links TRIM2, together with other factors, to the onset of neurodegenerative diseases, such as the Alzheimer disease (AD). The change seems to be directly mediated by the alteration of two specific miRNAs (miR9 and miR181c) in AD models (Schonrock et al., 2012).

As well as TRIM2, TRIM3 was also identified for the first time as interactor of myosin V in the rat brain (El-Husseini et al., 2001). Such interaction was further supported by a study on plasma membrane recycling: these two factors have been shown to interact with the endosome-associated protein Hrs and Actinin-4 in a complex named CART (cytoskeleton-associated recycling or transport), providing a first glimpse on the molecular mechanism for the actin-dependent recycling of plasma membrane receptors (Yan et al., 2005). Sequence analyses of *H. sapiens* genome allowed the identification of the human ortholog, located in the chromosome region 11p15, a locus frequently associated with several types of human cancer when deletions are present. TRIM3 was, thus, immediately regarded as a potential tumor suppressor (El-Husseini et al., 2001). Indeed, TRIM3 levels have been shown to be significantly decreased in hepatocellular carcinoma tissue samples, compared to healthy ones (Chao et al., 2014). But TRIM3 has mostly been studied in the context of brain tumor formation (Chen et al., 2014). For example, analyzing primary human gliomas, in around 25% of the studied cases, loss of heterozygosity in a genomic region containing a big deletion which comprises the *TRIM3* locus can be observed (Boulay et al., 2009). Furthermore, in mice, low levels of TRIM3 associate with a higher incidence and faster development of gliomas. The way TRIM3 is supposed to exert its role as a tumor suppressor is through the sequestration and inhibition of p21 which would prevent the entrance into the cell cycle (Liu et al., 2014). An *in vitro* study also confirmes the interaction of TRIM3 with p21, but clarifying that the cell growth suppression, mediated by TRIM3, is determined by its RING domain. Such domain has been shown to act as an E3 ligase, which, interacting with the E2 enzyme UbcH5a, promotes ubiquitination of p21 (Raheja et al., 2014). A role of TRIM3 as an E3 ligase was first proposed by a study which implicated it in mediating the degradation of postsynaptic density protein, specifically GKAP/SAPAP and Shank. The degradation of such factors has an important role in activity-dependent synaptic remodeling. Furthermore, the loss of TRIM3 does not only prevent such degradation, but it also seems to negatively affect the morphology of dendritic spines (Hung et al., 2010). Interestingly, a role in neuronal modeling and or signaling can be hypothesized as high levels of TRIM3 have been found in brains of patients with schizophrenia (Martins-Souza et al., 2009). Indeed, TRIM3 has been suggested to be a regulator of a neuronal kinesin (KIF21B) and, although no evidence for protein degradation has been found, the fact that the TRIM domain appears to mediate such function, mono-ubiquitination can be hypothesized as a mean to modulate the kinesin activity (Laboute et al., 2013). Finally, in a study where *TRIM3* was identified as a presumptive p53 target in human cells and mice, the TRIM3 protein has been shown to regulate the intracellular trafficking of $GABA_A$ receptors, most probably at a post-transcriptional level (Cheung et al., 2010).

4.2.4. TRIM32

TRIM32, initially called HT2A, is a human protein which was first identified as a mediator of the biological activity of the lentiviral Tat protein *in vivo* (Fridell et al., 1995). But TRIM32 has been mostly studied in the context of two unrelated human genetic diseases: depending on which domain of the protein is mutated the Bardet-Biedl syndrome (alteration in the BB1) and the limb girdle muscular dystrophy 2H (LGMD2H) (mutations in the NHL repeats) have been described. Interestingly, the same mutations causing LGMD2H have been also shown to cause sarcotubular myopathy (STM) (Tab. 1). The different etiology, based on what part of the protein is altered, suggests that TRIM32 utilizes different molecular mechanisms depending on which cellular or tissue context it is expressed. BBS is a ciliopathic disorder with pleiotropic effects and it is characterized by obesity, retinitis pigmentosa, polydactyly, hypogonadism and, in some cases, renal failure (Beales et al., 1999). LGMD2H and STM, instead, specifically affect muscles. In LGMD2H, the most severely affected muscles are the ones of hips and shoulders, which are characterized by weakness and atrophy. In in some cases, the disease can even cause cardiomyopathy (Nigro et al., 2011). The function of TRIM32 in LGMD2H has been the most studied one at a molecular level. In several studies, it has been demonstrated to act as an E3 ligase with different proposed specific substrates which would determine the onset and the worsening of the disease when not properly regulated (*e.g.*, actin (Cohen et al., 2012; Kudryashova et al., 2005), tropomyosin, troponins and α-actinin (Cohen et al., 2012), dysbindin (Locke et al., 2009) and c-Myc (Nicklas et al., 2012)). Interestingly, the creation of K.O. and knock-in (K.I.) mice (carrying the mutation D489N, corresponding to the human D487N) has been able to show that in both cases the observed phenotypes could recapitulate LGMD2H and STM (Kudryashova et al., 2009; Kudryashova et al., 2011). The fact that the K.O. and mutated K.I. protein can show the same phenotype has been demonstrated to be due to the reduction of the protein levels in the K.I. mice compared to the wild-type, suggesting that the observed phenotype is not due to the point mutation *per se* and making such model closer to a K.O. situation (Kudryashova et al., 2011). Controversially, a recent study on humans has analyzed two patients with full deletions of *TRIM32* in homozygosis. These patients present non-specific LGMD symptoms, suggesting that loss of TRIM32 does not associate with a specific phenotype (Nectoux et al., 2014). Finally, concerning the physiological functions of TRIM32, studies on cell culture and/or mice underline its importance for myogenic differentiation (Nicklas et al., 2012) and muscle regrowth after atrophy (Kudryashova et al., 2012).

TRIM32 has been also studied in a broader range of diseases and developmental contexts. In patients affected by psoriasis, TRIM32 has been found at high levels in epidermal lesions caused by aberrant regulation of keratinocytes (Liu et al., 2010). The E3 function of TRIM32 results important for the regulation of innate immunity against RNA and DNA viruses, as it was originally proposed when it was first discovered. TRIM32 targets for ubiquitination a host protein which could, otherwise, interact with a viral receptor (Zhang et al., 2012). High levels of TRIM32 also correlate with some neuronal disorders, such as AD (Yokota et al., 2006) and chronic stress-induced affective behaviors where TRIM32 is supposed to act as a regulator of hyperactive behavior, anxiety and depression disorders (Ruan et al., 2014). As other TRIM-NHL proteins, TRIM32 appears to be also involved in neuronal differentiation: an initial study in mouse, showed TRIM32 to be absent from neuronal progenitor cells, but present in differentiating ones: here, it ubiquitinates c-Myc for degradation and, through the binding to Argonaute-1, it increases *let-7* miRNA activity, required and sufficient for neuronal differentiation (Schwamborn et al., 2009). After such discovery, its role in neuronal differentiation has been then further analyzed in mice and cultured cell lines, finding relationships between TRIM32 with several factors, among which: the retinoic acid receptor α (as co-activator) (Sato et al., 2011), the protein kinase C ζ (sequestering TRIM32 in the cytoplasm) (Hillje et al., 2011), Staufen2 (Kusek et al., 2012), the transcriptional competent (ubiquitinating it) and the dominant negative (which inhibits *TRIM32* transcription) forms of p73 (Gonzalez-Cano et al., 2013).

On a final note, the understanding of which role TRIM32 plays in cancer is quite controversial. Studies in mice have shown TRIM32 to act as a tumor suppressor, whereas others as an oncogene. Its role in regulating differentiation of neuronal progenitor would hint for a general function as a tumor suppressor, but some evidences reported elevated levels of TRIM32 during murine carcinogenesis (Horn et al., 2004) and also in human leukemic cell lines (Sato et al., 2012). Different substrates and models have been proposed for TRIM32 acting as an E3 ligase in such context, for example as a negative regulator of apoptosis, blocking UVB-induced tumor necrosis factor α (TNFα) apoptotic signaling through controlling Piasy (a protein inhibitor of activated STATs) levels (Albor et al., 2006; Horn et al., 2004), or as a mediator of the degradation of the tumor suppressor Abl-interactor 2 (Abi2) (Kano et al., 2008) or p53, which seems, on its turn, to mediate *TRIM32* activation, through the binding to its promoter upon stress (Liu et al., 2014). On the contrary, as just mentioned, other more convincing studies show how TRIM32 acts as a tumor suppressor as a controller of asymmetric cell division in human cells (Izumi and Kaneko, 2014) and as it possesses pro-apoptotic functions (Ryu et al., 2011).

Protein	Affected domain	Disease	Reference
P130S	BB1	BBS type 11	*Chiang et al., 2006*
H452T	NHL	Schizophrenia	*Farous et al., 2004*
D487N	NHL	STM	*Schoser et al., 2005*
D487N	NHL	LGMD2H	*Frosk et al., 2002; Frosk et al., 2005*
R394H	NHL	LGMD2H	*Saccone et al., 2008*
T520TfsX13	NHL	LGMD2H	*Saccone et al., 2008*
D588del	NHL	LGMD2H	*Saccone et al., 2008*
I590LfsX38	NHL	LGMD2H	*Coss et al., 2009*
R136Stop	From BB1	LGMD2H	*Neri et al., 2013*
full deletion	all	non specific LGMD2H	*Nectoux et al., 2014*

Table 1. Genetic diseases correlating with mutations and affecting different domains of TRIM32: List of all the identified mutations (at a protein and domain level) for TRIM32 and the related human diseases they determine.

4.2.5. LIN-41/TRIM71

LIN-41 (LINeage defective 41), also known as TRIM71, was first identified in *C. elegans* as a member of the so-called heterochronic pathway and speculated to be acting as a post-transcriptional regulator (see next section). Specifically, its mRNA was identified as a direct target of the tumor suppressor miRNA *let-7* (Reinhart et al., 2000; Slack et al., 2000), able to inhibit *lin-41* translation and to promote its degradation thank to the presence of two *let-7* binding sites (LBSs) in the 3'UTR of the transcript (Vella et al., 2004). Not only the two genes results to be conserved in vertebrates, but also the *let-7*-mediated regulation of *lin-41* expression through LBSs (Kanamoto et al., 2006; Lancman et al., 2005; Lin et al., 2007; Schulman et al., 2005). For these reasons, for several years *lin-41* has not been studied for its biological roles, but simply as a tool to study and to measure the *let-7* activity as a miRNA. From the first studies on different organisms, it immediately appeared clear that LIN-41 can be considered a temporal regulator which changes its spatial expression patterns over developmental times (Lancman et al., 2005; Schulman et al., 2005; Slack et al., 2000; Yu et al., 2010). More specifically, it has been shown to promote cell proliferation and prevent cell differentiation, playing key roles not only during early embryonic development, but also during carcinogenesis. Concerning its roles in the embryo, it has been proved to be involved in chick and mouse limb development (Lancman et al., 2005), mouse embryonic survival and neural tube closure (Maller Schulman et al., 2008), proper timing in zebrafish embryonic development (Lin et al., 2007) and promoting mouse neuronal progenitor maintenance, preventing their precocious differentiation (Chen et al., 2012). In disease, high levels of LIN-41 have been reported in hepatocellular carcinoma (Chen et al., 2013) and, among other proteins, in myxoid

liposarcoma (De Cecco et al., 2014). Furthermore, LIN-41 activity is not restricted to undifferentiated cells, but does also seem to be involved in post-differentiation events, such as axon regeneration in worms. Its action is restricted to growing neurons, as *let-7* gets expressed in such terminally differentiating cells, therefore inhibiting LIN-41 function and promoting the expression of the TF LIN-29 (Zou et al., 2013). Given its role in preventing cell differentiation, it is not fully surprising that, recently, LIN-41 has been proved to be able to substitute c-Myc to create iPS cells when transfected into fibroblasts together with Oct4, Sox2 and Klf4 (Worringer et al., 2014). Considering that a previous study could show the direct interaction of c-Myc with the *lin-41* promoter, enhancing its expression (Chen et al., 2013), LIN-41 can be, then, considered the main, if not the only, effector of c-Myc in the context of iPS cells. *let-7* and c-Myc, which respectively negatively and positively regulate LIN-41 expression levels, are not the only proposed regulators: LIN-41 has been reported to act downstream the FGF signaling pathway (Lancman et al., 2005), although in a more recent study, it appears to simply mediate the proper activity of such pathway (Chen et al., 2012). Specifically, LIN-41 has been suggested to act as an E3 ligase and to mediate the ubiquitination and the stabilization of Shc SH2-binding protein 1 (SHCBP1), one of the effector of the FGF pathway.

LIN-41 is a cytoplasmic protein, enriched in P-bodies (Chang et al., 2012; Rybak et al., 2009): such subcellular localization suggests a candidate role for it as an RBP acting in post-transcriptional gene regulation. This role was, indeed, confirmed by studies on ES cells (Chang et al., 2012; Kwon et al., 2013) and a miRNA-dependent interaction with Dicer and different Argonaute proteins was shown to enhance the miRNA pathway activity (Chang et al., 2012; Zou et al., 2013). Chang and co-workers have nicely proposed that LIN-41 interacts in a miRNA-dependent manner with Argonaute 2 (Ago2), through the NHL-mediated binding to the two miRNAs miR-302 and miR-290, and cooperates with it in promoting ES cells proliferation. Altogether, these factors can control the cell cycle by repressing the translation of the *Cdkn1a* mRNA, a cell cycle regulator belonging to the p21 pathway (Chang et al., 2012). The presence of LIN-41 in P-bodies has been differently interpreted in another study, where LIN-41 has been shown to interact with different component of the RISC complex in HeLa and EC cells and to act as an E3 ligase *in vitro* ad *in vivo*, interfering, in this case, with the miRNA activity. Specifically, LIN-41 is shown to bind the E2 conjugating enzyme UbcH5a to ubiquitinate Ago2, thank to its CC-mediated binding. In such way, LIN-41 cooperates with LIN28 in suppressing *let-7* activity, providing an additional layer of regulation in stem cells (Rybak et al., 2009). The LIN-41-dependent ubiquitination and the following degradation of Ago2 has not be confirmed in another study, although the E3 activity has (Chen et al., 2012). Despite that, an additional support to the hypothesis that LIN-41 antagonizes miRNA activity has been provided later on

with indirect evidences concerning the changes in the levels of LIN28B and c-Myc, both suppressors of *let-7*, accordingly to the changes in LIN-41 and *vice versa* (Chen et al., 2013). Controversially, it has also been proposed that LIN-41 is acting exactly in the opposite way of what has just been described: LIN-41 seems to ubiquitinate LIN28B, allowing *let-7* to be properly processed and to reach its mature form (Lee et al., 2014). This hypothesis would suggest a model where the three factors balance their expressions inhibiting each other in a feed-back loop. Although interesting, this hypothesis goes against empirical evidences which demonstrate that *let-7* and LIN-41 are mutually exclusive and control cell fate decision.

Given the fact that *in vitro* and *in vivo* studies have been able to demonstrate the E3 ligase properties of LIN-41, only in the last couple of years more attention has been paid to the possible NHL-mediated binding to RNAs and their consequent role in post-transcriptional gene regulation. Some example has already been discussed, but the major contribution has for sure been provided by Loedige and co-workers. The target transcripts bound and, presumably, regulated by LIN-41 have been identified and, together with them, reporter assays have been able to demonstrate their post-transcriptional gene silencing mediated by the binding of LIN-41 to their 3'UTRs (Loedige et al., 2013). In addition, analyses with truncated versions of the LIN-41 protein have been able to show the roles of its different domains in post-transcriptional gene regulation: from the TRIM domain, only the CC, together with the filamin, seems to be essential in recruiting the additional components which mediate the actual mRNA silencing, whereas the NHL repeats alone mediate the protein-mRNA interaction. Other functional studies have been made in a previous work and they could also show the importance of the CC in post-transcriptional gene silencing and a direct interaction of the NHL repeats with RNAs (miRNAs). Furthermore, they have also been able to provide some additional information about the subcellular localization of LIN-41, which seems to be mediated by BBs and NHL repeats (Chang et al., 2012). The list of transcripts bound by LIN-41 (Loedige et al., 2013), together with the wild-type versus *lin-41* knock-down transcriptional profiles (Worringer et al., 2014) and the putative interacting partners of LIN-41 (Yu et al., 2010) can provide a good baseline to interpret LIN-41 function in development as an RBP and not only as an E3 ligase.

5. LIN-41/TRIM71 and the heterochronic pathway in *Caenorhabditis elegans*

As already mentioned, LIN-41 was first identified in the context of the worm heterochronic pathway. Such pathway consists of a cascade of regulatory genes which are temporally controlled to

specify the timing of developmental events (Ambros, 1989). More specifically, these genes control the cell fate (proliferation versus differentiation) of certain worm epidermal cells, the seam cells: mutations in any of the genes belonging to the pathway would cause an alteration of the peculiar stage-specific events and determine either a precocious differentiation or a prolonged proliferation (Fig. 12) (Ambros and Horvitz, 1984; Reinhart et al., 2000).

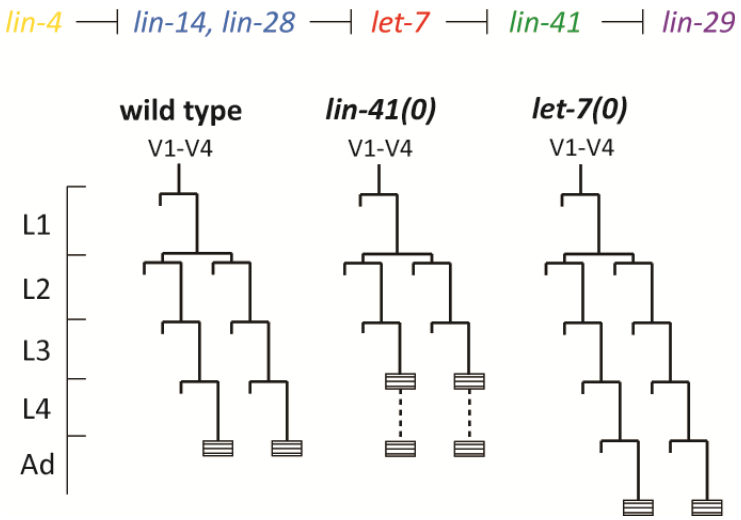

Figure 12. ***C. elegans*** **somatic heterochronic pathway – members and phenotypes (adapted from Reinhart et al., 2000 and Slack et al., 2000)**: Upper part: members of the heterochronic pathway. Lower part: cell lineages of the lateral hypodermal V cells. Between every larval stage (y axis), at the molting time, the V cells divide in a stem cell-like fashion until the L4-to-adult molt, when they terminally differentiate (striped box) in the wild-type situation. In the *lin-41* mutant (*lin-41(0)*), they precociously differentiate one molting cycle before the wild-type. In the *let-7* mutant (*let-7(0)*), instead, they divide once more after reaching adulthood. After this extra-division these cells terminally differentiate.

This pathway has been extensively studied to show how miRNAs (*lin-4* and *let-7*) can finely regulate cell fate decision in the physiological development of an organism. From these studies, a clear model on how the different members of the pathway temporally control each other has been described (Fig. 13), but for many years not much attention has been given to the specific and additional roles of its downstream effectors, such as LIN-41 and LIN-29. For example, different phenotypic defects have been actually observed in *lin-41* null animals (*e.g.*, sterility (Slack et al., 2000)), but they have never been taken into account. Only after some years from the first study on LIN-41, a not really convincing work started analyzing its function aside of the seam cell development, showing that two presumptive gain of function alleles for this gene cause a heterochronic delay in the context of the worm mail tale tip morphogenesis (Del Rio-Albrechtsen et al., 2006). Apart from this study, no further insights on LIN-41 functions have been done. Despite the fact that a direct evidence has never been provided, based on mRNA and protein expression profiles, LIN-41 has always been regarded as an RBP, supposed to bind the *lin-29* transcript and to negatively regulate its expression. Such inhibition would only get relived

when LIN-41 is down-regulated by *let-7*, during the L4 to adult molt (Fig. 13). In case LIN-41 is, indeed, acting as a post-transcriptional regulator, one could hypothesize that an additional candidate mRNA target could be represented by *mab-10*, whose expression pattern somehow reflects the one of *lin-29* and together with it coordinates some aspects of the L4 to adult transition (Harris et al., 2011).

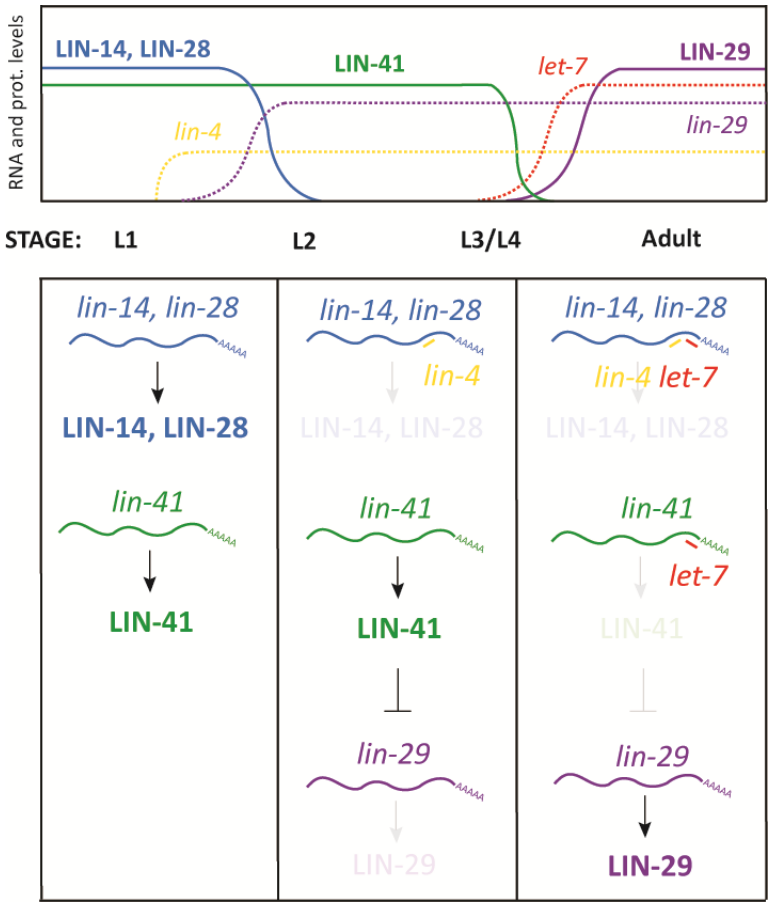

Figure 13. ***C. elegans*** **somatic heterochronic pathway – protein and mRNA levels and mechanism (adapted from Reinhart et al., 2000)**: Described model for the successive post-transcriptional regulation of the members of the heterochronic pathway. Upper panel: model describing how RNA and protein levels (y axis) of the members are changing over developmental time (x axis). Lower panel: model describing the mechanisms through which the single members post-transcriptionally affect the next over developmental time (x axis). Note that *lin-4* and *let-7* are miRNAs.

It is clear, then, that no sufficient efforts have been put in understanding how LIN-41 molecularly functions and in which other biological contexts and pathways can act in the physiological development of an organism. Studies on LIN-41 and its orthologs suggest a candidate role as an oncogene, acting both as an E3 ubiquitin ligase and RBP, but if this is the standard or just one among the possible scenarios, it has to be demonstrated.

Results

1. Manuscript:

"The TRIM-NHL protein LIN-41 controls the onset of developmental plasticity in *Caenorhabditis elegans*"

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SHORT TITLE

LIN-41 controls developmental plasticity

ABSTRACT

The mechanisms controlling cell fate determination and reprogramming are fundamental for development. A profound reprogramming, allowing the production of pluripotent cells in early embryos, takes place during the oocyte-to-embryo transition. To understand how the oocyte reprogramming potential is controlled, we sought *Caenorhabditis elegans* mutants in which embryonic transcription is initiated precociously in germ cells. This screen identified LIN-41, a TRIM-NHL protein and a component of the somatic heterochronic pathway, as a temporal regulator of pluripotency in the germline. We found that LIN-41 is expressed in the cytoplasm of developing oocytes, which, in *lin-41* mutants, acquire pluripotent characteristics of embryonic cells and form teratomas. To understand LIN-41 function in the germline, we conducted structure-function studies. In contrast to other TRIM-NHL proteins, we found that LIN-41 is unlikely to function as an E3 ubiquitin ligase. Similar to other TRIM-NHL proteins, the somatic function of LIN-41 is thought to involve mRNA regulation. Surprisingly, we found that mutations predicted to disrupt the association of LIN-41 with mRNA, which otherwise compromise LIN-41 function in the heterochronic pathway in the soma, have only minor effects in the germline. Similarly, LIN-41-mediated repression of a key somatic mRNA target is dispensable for the germline function. Thus, LIN-41 appears to function in the germline and the soma via different molecular mechanisms. These studies provide the first insight into the mechanism inhibiting the onset of embryonic differentiation in developing oocytes, which is required to ensure a successful transition between generations.

AUTHOR SUMMARY

Reprogramming into a naïve, pluripotent state during the oocyte-to-embryo transition is directed by the oocyte cytoplasm. To understand how this reprogramming is controlled, we searched for *C. elegans* mutants in which the activation of embryonic genome, a landmark event demarcating the switch from a germline- to embryo-specific transcription, is initiated precociously in germ cells. This screen identified a novel function for LIN-41, a member of the TRIM-NHL protein family, in preventing a premature onset of embryonic-like differentiation and teratoma formation in developing oocytes, thus ensuring a successful passage between generations. This is the first example of such a regulator in cells that are poised for embryonic development. Interestingly, the majority of molecular "roadblocks" to reprograming that have been identified so far are epigenetic regulators. However, we propose that, at least in germ cells, LIN-41-like regulators may fulfill an analogous role in the cytoplasm, which has possible implications for the generation of human pluripotent stem cells.

INTRODUCTION

There is a special relationship between germ cells and pluripotency, *i.e*,. the ability to adopt alternative cell fates. First, germ cells transmit the pluripotent potential to recreate all types of cells in a new individual. Second, germ cells give rise to pluripotent cell lines such as embryonic germ or carcinoma cells and oocyte cytoplasm has the capacity to reprogram somatic nuclei [1,2]. Finally, in disease, germ cells can abnormally differentiate into diverse somatic cell types, forming teratomas. However, during normal development, the ability to differentiate into all three embryonic germ layers is restricted to the cells of the early embryo. Combined, these observations suggest that the reprogramming potential of germ cells is kept at bay by repressive mechanisms. Depletion of several chromatin modifiers, either alone or combined with an ectopic overexpression of somatic cell fate-specifying transcription factors, can induce reprogramming of *C. elegans* germ cells into somatic cells [3-5]. The loss of these factors appears to primarily impact proliferating (pre-meiotic) germ cells and affects chromatin-based regulation. In contrast, our previous work in the same animal demonstrated that a conserved RNA-binding protein, GLD-1/Quaking, prevents teratomatous differentiation of post-mitotic germ cells [6,7]. Importantly, in *gld-1* mutants, the germline-to-soma transition is accompanied by a precocious onset of embryonic (or zygotic) genome activation (EGA), suggesting a causal connection between EGA and pluripotency. In other animals, the connection between EGA and pluripotency has been also postulated based on the temporal correlation between EGA and the acquisition of a pluripotent chromatin landscape [8,9].

These observations prompted us to examine whether new regulators of pluripotency can be identified based on a precocious onset of EGA in the germline. Here, we report the discovery of one such novel regulator of pluripotency, LIN-41/TRIM71. LIN-41 belongs to the TRIM-NHL protein family [10]. These proteins contain a TRIpartite Motif (TRIM) consisting of a RING finger domain (commonly endowing a protein with E3 ubiquitin ligase activity, for example [11-13]), two B-Box motifs and a coiled-coil domain. Additionally, they also carry six so-called NHL repeats (named after *N*CL-1, *H*T2A and *L*IN-41) and may contain a filamin domain, which have been implicated in both protein-protein and protein-RNA interactions [13-17]. Consistently, different molecular functions have been attributed to LIN-41-like proteins, but many questions remain open; for example, it is not clear whether all the domains function together and/or are used in a tissue context-dependent manner [11,14,18-20]. The TRIM-NHL family includes well-known regulators of self-renewal and differentiation. For example, in *Drosophila melanogaster,* Brat inhibits neuroblast self-renewal, cell growth and ribosome synthesis in the larval

brain [21-24] and Mei-P26 restricts growth and proliferation in the ovarian stem cell lineage [25]. Defects in TRIM-NHL proteins have also been associated with human pathologies, for example TRIM32 has been implicated in the Bardet–Biedl Syndrome and the Limb-Girdle Muscular Dystrophy [12,26,27]. Recently, human LIN-41 has been shown to promote reprogramming of differentiated cells into induced pluripotent stem cells (iPSCs) [28]. Here, we demonstrate a role for LIN-41 in controlling pluripotency during development of an animal. In *C. elegans*, LIN-41 is a well-known component of the somatic heterochronic pathway, which temporally controls the transition from larval to adult cell fates [29,30]. The *lin-41* germline phenotype described here indicates that, by preventing the onset of embryonic events in developing oocytes, LIN-41 also ensures a successful transition between generations. However, based on our analyses on both existing and newly created LIN-41 mutations, LIN-41 appears to function in the germline and the soma via two distinct molecular mechanisms. Our study identifies the first cytoplasmic "molecular roadblock" to reprogramming in developing oocytes and we propose it to be required to delay the onset of embryonic differentiation until after fertilization.

RESULTS

To understand how the onset of pluripotency is controlled during *C. elegans* development, we executed a genetic screen to identify factors that prevent EGA in the adult germline. To monitor EGA, we created a strain expressing GFP from an early embryonic promoter, *vet-4* (very early transcript 4) [31,32]. Thus, to identify novel regulators of developmental plasticity, we searched for mutants expressing the EGA-GFP in the adult germline (Figure 1A). In addition to a new allele of *gld-1*, this screen yielded two mutants that, in contrast to the embryo-specific EGA-GFP expression in wild-type animals, expressed EGA-GFP within the gonads (Figure 1B). Several lines of evidence suggested that the phenotype of the two mutant strains was caused by alterations in the same gene, *lin-41* (Figures 1C and S1A-D). In these mutants (alleles *rrr3* and *rrr4*, Figure 1C), the EGA-GFP expression was restricted to the proximal region of the oogenic germline (Figure 1B). Consistent with this, RNAi-mediated depletion of *lin-41* resulted in a similar expression of EGA-GFP in the gonad (Figure S1C) and a transgenic construct expressing LIN-41 fully rescued the germline defects of *lin-41(rrr3)* animals (Figure S1D). To further examine the role of LIN-41 in controlling EGA, we verified that the endogenous *vet-4* is also abnormally transcribed in *lin-41(rrr3)* gonads. Indeed, by *in situ* hybridization, we could detect *vet-4* to be expressed in the proximal gonads of *lin-41(rrr3)*, but not wild-type animals (Figure 1D). Next, to examine the extent of embryonic-like transcription in *lin-41(rrr3)* gonads, we monitored the levels of *vet-4* and other additional early embryonic transcripts by reverse transcription and quantitative PCR (RT-qPCR) (Text S1). We found that these transcripts were expressed in mutant, but not wild-type gonads (Figure 1E), further demonstrating that, in *lin-41* mutants, embryonic transcription is prematurely activated in the germline. Importantly, we detected no obvious changes in levels or expression pattern of GLD-1 in *lin-41(rrr3)* gonads (Figure S2), suggesting that the gonadal phenotype of *lin-41* mutants is not caused by defective expression of GLD-1.

In wild-type animals, Pol II-dependent transcription is repressed in oocytes, which is seemingly at odds with the embryonic-like transcription in the proximal gonads of *lin-41* animals. To investigate this potential discrepancy, we examined the transcription-initiating phosphorylation of serine 5 (Ser5P) within the C-terminal domain (CTD) of Pol II [33]. In contrast to wild-type gonads, Ser5P was detected in the majority of the cells in the proximal gonads of *lin-41(rrr3)* animals (Figure 2A), indicating ongoing Pol II-dependent transcription. Apart from EGA, the onset of embryonic development is marked by the degradation of germline mRNAs and proteins [34]. To examine this aspect of the germline-to-soma transition in *lin-41* animals, we followed the expression levels of RME-2, a yolk receptor present in

oocytes [35], and PGL-1, a constitutive component of germ cell-specific RNA/protein granules [36]. In contrast to wild-type animals, which express RME-2 in developing oocytes and PGL-1 throughout the germline, we found that both proteins were absent from the proximal *lin-41(rrr3)* gonads (Figure 2B-C), indicating that cells in this gonadal region lose germline identity. To test this further, we monitored expression of several transcripts that are normally expressed in somatic lineages. By RT-qPCR (Text S1), we found that several of these transcripts (for example the myogenic *hlh-1*/MyoD) were abnormally expressed in *lin-41(rrr3)* gonads (Figure 2D). Additionally, we examined the expression of several *hox* genes, which control the positional identities of cells during animal body formation [37]. While the *hox* transcripts were not expressed in wild-type gonads, they were strongly expressed in *lin-41(rrr3)* gonads (Figure 2D). Finally, we analyzed the expression of the muscle lineage markers UNC-120 and muscle myosin, the intestine lineage marker ELT-2 and a GFP reporter driven from a pan-neuronal *unc-119* promoter (nGFP). We observed that *lin-41(rrr3)* gonads contained numerous cells expressing muscle and neuronal markers (Figures 2E and S3; 44/45 examined gonads contained cells expressing UNC-120, 10/18 cells expressing muscle myosin, and 57/57 cells expressing the nGFP). Only few gonads contained ELT-2-expressing cells (3/35 gonads and only in few cells), which might reflect a competitive advantage of some differentiation programs in the *lin-41* teratoma. During embryogenesis, most body-wall muscles of an adult animal are specified by the transcription factor PAL-1/CDX [38]. The PAL-1-dependent transcription is relatively well understood and involves the activation of its direct targets, such as HLH-1/MyoD and UNC-120/SRF [39]. In wild-type oocytes, expression of PAL-1 is insufficient for the induction of its target genes (Figure S4A-B) [40]. Nevertheless, we observed that the numbers of UNC-120-expressing cells in *lin-41(rrr3)* gonads were significantly reduced upon *pal-1* RNAi (Figure S4B). Thus, the differentiation into muscles in *lin-41* gonads appears, at least partly, to mimic the pathway driving muscle formation in embryos. Together, these findings indicate that *lin-41* germ cells in the proximal gonad undergo a dramatic reprogramming, which results in the acquisition of an embryonic-like state and teratomatous differentiation.

To better understand the germline-to-soma transition in *lin-41* animals, we examined cells in *lin-41(rrr3)* gonads in a time-course experiment (Figure 3A). Until immediately after the end of spermatogenesis, the morphology and numbers of germ cells in *lin-41* and wild-type gonads appeared similar. However, concomitantly with the onset of oogenesis, differences between the *lin-41* and wild-type germlines began to emerge. The proximal region of wild-type gonads contained fully-grown oocytes harboring chromosomes arrested at the diakinesis stage of meiosis I. In stark contrast, the proximal region of *lin-41* gonads contained oocyte-like cells that were about to divide, as evidenced by

the presence of highly condensed chromosomes (marked by the phosphorylation of histone H3 on serine 10, Ser10P [41], and microtubule spindles (Figure 3A-B). Consistent with entering a mitotic cell cycle, cells in the proximal *lin-41(rrr3)* gonads did not express HIM-3 (Figure 3C), a synaptonemal complex component [42]. Wild-type oocytes eliminate centrosomes, presumably to ensure the correct ploidy in embryos [31,43,44]. In contrast, by monitoring a constitutive centrosome component, SPD-2 [45], we found that centrosomes were present in the proximal *lin-41(rrr3)* gonads (Figure S5). These centrosomes could duplicate (Figures 3D and S5) and were able to nucleate microtubule spindles (Figure 3D). Finally, in addition to the cell cycle markers, we monitored expression of an EGA reporter (EGA-mCherry) and the muscle-lineage marker UNC-120 and observed that their expression followed the onset of mitosis (Figure 3A). Taken together, the absence of LIN-41 leads to the elimination of germline proteins, induction of EGA, a change from the meiotic to the mitotic cell cycle and somatic-like differentiation. Thus, rather than completing oogenesis, cells in the proximal *lin-41* gonads execute events that, in wild-type development, only occur during embryogenesis.

In addition to the germline defects, *lin-41(rrr3)* animals displayed somatic abnormalities: decreased size (dumpy phenotype), appeared sick and occasionally bursted through the vulva. These phenotypes have been extensively described by Slack and colleagues and are caused, at least in part, by a precocious translation of the transcription factor LIN-29 [29]. To determine whether the gonadal phenotype reflects LIN-41 function in the germline, or it is indirectly caused by the loss of LIN-41 in the soma, we created a transgene driving *lin-41* expression from a heat-shock promoter (*hsp-16.41*). Due to a general insensitivity of germ cells to the heat-shock promoter-driven expression [46], this transgene was not expressed in the germline but, when crossed into the *lin-41(rrr3)* mutant background and cultivated at an elevated temperature (24-25°C, which is apparently enough to drive sufficient expression of *lin-41* in the soma), it rescued the somatic *lin-41* defects (the transgenic animals no longer appeared sick or short; Figure 4A). Despite the somatic rescue, these animals still developed teratomas (Figure 4B, 50/50 examined animals), suggesting that, in controlling the germline-to-soma transition, LIN-41 functions autonomously in the germline. To examine this further, we immunostained gonads using antibodies raised against LIN-41 and found that, indeed, LIN-41 was present in the cytoplasm of germ cells starting from the late pachytene stage and culminating in the fully-grown oocytes (Figure 4C). Interestingly, LIN-41 was often absent from the most-proximal oocytes (Figures S1D and S6), suggesting a possible connection between oocyte maturation and/or ovulation and LIN-41 levels. LIN-41 expression was limited to the oogenic germline (*i.e.*, it was absent in sperm, *e.g.*, S1D), suggesting that the

germline-to-soma transition in *lin-41* gonads is caused by the loss of LIN-41 function in the developing oocytes.

In the soma, LIN-41 is thought to associate with and repress the mRNA encoding a transcription factor, LIN-29, and LIN-29 depletion suppresses the somatic defects of *lin-41* mutants [29]. In contrast to these observations in the soma, *lin-29* mRNA appears to be either poorly or not at all expressed in the germline (our unpublished results and [47]). Consistently, we found that RNAi-mediated depletion of LIN-29 did not suppress the germline defects of *lin-41(rrr3)* mutants (though, it suppressed the somatic defects, as expected [29]). We obtained similar results in *lin-41; lin-29* double mutants (Figure S7). Thus, LIN-41 may function in the germline and soma via distinct targets and/or mechanisms.

The domain structure of LIN-41 reflects the diversity of functions that have been associated with TRIM-NHL proteins (Figure 1C). Several of these proteins function as E3 ubiquitin ligases, which require a functional RING domain [10,30]. However, a sequence alignment of the *C. elegans* LIN-41 RING domain with those of other *Caenorhabditis* species indicates that a highly conserved proline, critical for canonical E3-E2 interactions [48], is not found in the nematode LIN-41 RING domains (Figure 5A). Moreover, mutating five cysteine residues that are critical for the RING domain zinc finger structure (C114S, C117S, C130S, C151S, C154S; Figure 5A) resulted in a protein that rescued both somatic and germline defects of *lin-41(rrr3)* animals (Figure 5B). Although we cannot rule out the possibility that LIN-41 associates with additional factors to regulate ubiquitination, these results suggest that the nematode LIN-41 does not function as a direct E3 ubiquitin ligase.

The coiled-coiled, filamin and NHL domains of the human homolog of LIN-41, TRIM71, constitute the minimal region responsible for binding mRNA and inhibiting translation [19], which is the function attributed to the *C. elegans* LIN-41 in the soma [29]. One previously isolated *lin-41* allele, *ma104*, is a transposon insertion into the sequence coding for the filamin domain [29]. Intriguingly, *lin-41(ma104)* mutants display the somatic defects, but the animals are apparently fertile [29]. We confirmed this and, although the brood size in *lin-41(ma104)* animals is decreased [29], we found no obvious differences in the levels or localization of the germline LIN-41^{ma104} (Figure 6A) and no evidence for a precocious EGA in the gonads (0/35 worms were expressing the EGA reporter in their gonads). To understand the effect of the *ma104* mutation on the LIN-41 protein, we examined the cDNA product of the *lin-41(ma104)* allele. We found that the *ma104* mutation resulted in an insertion of 16 amino acids into the filamin domain (Figure 6B). To gain a mechanistic insight into the *ma104* mutation, the filamin domain (residues 691-821) was subcloned, overexpressed in bacteria, and the protein purified to homogeneity. The protein

was crystallized and the structure determined at high resolution (1.68 Å; for data collection and refinement statistics, see Table S1). The final crystallographic model encompasses residues 691-729 and 758-820, whereas a long insert (730-757) that is only found in *Caenorhabditis* LIN-41 protein sequences could not be built due to high flexibility. We found that the LIN-41 filamin structure exhibits a classical immunoglobulin (IG)-like domain fold consisting of seven □-strands arranged in two antiparallel □-sheets (Figure 6C) [49]. A structural search with the LIN-41 filamin domain against the Protein Data Bank (PDB), using DALI, identified the filamin domains most structurally similar to that of LIN-41, which yielded the filamin domains from the *Dictyostelium discoideum* gelation factor, the human TRIM45 and Filamin-A (PDB IDs 1QFH, 1WLH, 2DS4 and 3RGH, respectively) with root-mean-square deviation values for C□ positions between 1.6 and 2.0 Å [50]. Both crystal packing analysis using PISA [51] and SEC MALS experiments of the protein in solution reveal the oligomeric state of this protein domain as monomeric. Importantly, the 16-residue insertion present in the *ma104* allele maps to the mid-section of the second □-strand and is very likely perturbing the filamin IG-like fold (Figure 6B-C). Specifically, the 16-residue insert will prevent completion of one of the □-sheets resulting in solvent access to the hydrophobic protein core of the IG-like □-sandwich and thereby severely destabilizing the fold. This makes it very unlikely that the filamin domain of the *ma104* allele is properly folded to exert its biological function.

In the fly Brat and the mammalian TRIM71/LIN-41, the NHL domain is essential for mRNA regulation [14,19,52]. One of the *lin-41* alleles reported here, *rrr4*, introduces a premature stop codon within the first NHL repeat (Figure 1C), potentially triggering mRNA degradation via nonsense-mediated mRNA decay (NMD). Indeed, inhibiting NMD (by depleting an NMD component, SMG-2 [53]), restored the wild-type expression pattern and levels of LIN-41^{rrr4} (Figure 7A). LIN-41^{rrr4} is expected to lack the NHL domain and we found that the gonads expressing this LIN-41 variant displayed *lin-41*-like germline and somatic defects (Figure 7A). Thus, the NHL domain appears to be essential for LIN-41 functions in both germ and somatic cells.

The NHL domain structure of Brat forms a six-bladed □-propeller [52]. Several point mutations in Brat and TRIM71 that disrupt mRNA regulation affect residues on the electropositive side of the NHL domain [19,23,52,54] (Figures S8-9), highlighting the importance of this surface for mRNA regulation. Point mutations in the NHL domain have been also reported in LIN-41 (Figures 7B and S9A-B) [29]. Importantly, although these mutations display defects in the soma, the animals are fertile, suggesting that the mutant proteins fulfill the gonadal functions [29]. To better interpret these mutations, we initially attempted, unsuccessfully, to express the LIN-41 filamin-NHL or a NHL-only domain constructs

for protein structure determination. Thus, we created a homology model of the LIN-41 NHL domain based on the crystal structure of the Brat NHL domain. Interestingly, we found that most of the existing point mutations in the LIN-41 NHL domain also affect amino acids residing on the electropositive surface of the NHL domain (Figure 7B). These observations suggest that i) the electropositive surface of the NHL domain plays a conserved function in mRNA regulation and ii) the germline and somatic functions of the NHL domain involve different mechanisms. To explore this further, we introduced an additional mutation (Y941A) on the electropositive surface of the NHL domain (Figures 7B and S9). Potentially, this mutation is more informative than the other existing NHL mutations because mutation of the corresponding residue (Y702A) in TRIM71 is known to abolish mRNA regulation [19]. In contrast to the deletion of the whole NHL domain from otherwise rescuing (FLAG- and GFP-tagged) LIN-41 protein, which, as expected, caused defects in both the soma and the germline, we found that the LIN-41^{Y941A} variant largely suppressed the germline defects and sterility of *lin-41* animals (Figure 7C), including the precocious expression of the endogenous *vet-4* transcript (Figure 7D), though it continued to display the somatic defects. Thus, if the same domains/residues determine mRNA regulation in LIN-41 as in TRIM71, the germline function of LIN-41 might be independent from mRNA binding.

DISCUSSION

Cytoplasmic regulators of pluripotency

In contrast to the much-publicized regulation of pluripotency via DNA and chromatin modifications, the potential for cytoplasmic regulation has largely been neglected. Our findings suggest that proteins like LIN-41 and GLD-1 can function in the cytoplasm as molecular "roadblocks" to reprogramming, analogous to the nuclear factors. Similarly, components of P-granules (germline-expressed RNPs) have been recently reported to facilitate maintenance of germline identity in proliferating germ cells, *i.e.*, at the stage prior to GLD-1 expression [55]. Whether teratomatous differentiation in the absence of P-granules reflects precocious activation of embryonic transcription, or has a different etiology, remains to be determined. However, in contrast to P-granules, that impact multiple aspects of RNA metabolism, GLD-1 and LIN-41 are expected to have more specific functions. Intriguingly, the germline-to-soma transition in the absence of LIN-41 or GLD-1 involves similar events: loss of germline proteins, retention of centrosomes, execution of mitosis and activation of the embryonic genome. Two GLD-1 mRNA targets, important for the germline-to-soma transition, encode the CDK-2 partner protein CYE-1/cyclin E and the transcription factor PAL-1/Cdx [6,31]. However, cyclin E and PAL-1 are co-expressed with LIN-41 in the developing wild-type oocytes [56], suggesting that their expression is not regulated by LIN-41. Thus, GLD-1 and LIN-41 may regulate pluripotency via different targets and/or mechanisms. While GLD-1 directly binds and regulates the expression of its mRNA targets, the molecular function of LIN-41 remains elusive. Our analysis suggests that the germline function of LIN-41 may be independent from mRNA binding, though it does not exclude its role in posttranscriptional regulation, for example as a component of a regulatory RNP. In addition to binding RNA, several TRIM-NHL proteins have been shown to modulate functions of other proteins, for example, by their sequestration (*e.g.*, TRIM3 appears to regulate p21 [13,17]) or by linking structural proteins (*e.g.*, Wech bridges Talin and ILK for proper embryonic muscle attachment [16]). These interactions depend, at least in part, on the NHL domains of both proteins. Thus, LIN-41 could regulate the germline-to-soma transition by associating, via its NHL domain, with another protein.

Temporal regulation of the germline-to-soma transition by LIN-41

LIN-41-mediated regulation of cell fate transition between generations is somewhat reminiscent of LIN-41 function in the hetrochronic pathway in the soma. However, specific mutations within the NHL and filamin domains of LIN-41 result mainly in somatic but not germline defects (this study and [29]). In

addition, LIN-41-dependent repression of LIN-29 appears to be restricted to the soma. Thus, although LIN-41 regulates developmental transitions in both germ- and somatic cells, it may do so through different molecular mechanisms and/or targets.

In the soma, down-regulation of LIN-41, which is mediated by the *let-7* miRNA, allows terminal differentiation [57,58]. In the germline, LIN-41 levels decrease in the most-proximal oocytes, so that LIN-41 is absent from the early embryos. An interesting possibility is that the absence of LIN-41 is a trigger for the onset of embryonic differentiation. In order to test this hypothesis, we attempted to over-express LIN-41 from the heat shock promoter in very early embryos. However, LIN-41 was efficiently expressed only after gastrulation (our unpublished observation), *i.e.*, several cell divisions after EGA, making the experiment inconclusive. The down-regulation of LIN-41 occurs while Pol II-dependent transcription is globally repressed in the oocytes, suggesting that the regulation occurs at the mRNA or the protein level. To test for possible regulation at the mRNA level, we expressed a rescuing LIN-41 under the control of a truncated 3'UTR missing most of the sequence, including the *let-7* binding sites [58]. While the expression of this LIN-41 protein started earlier (more distally) in the germline, suggesting posttranscriptional regulation of *lin-41* mRNA in this part of the gonad, LIN-41 was still down-regulated in the oocytes and embryos (our unpublished observation), hinting at a possible regulation at the protein level. If so, testing the functional significance of LIN-41 degradation will require the dissection of regulatory motifs in the protein (for example phosphorylation).

Mammalian LIN-41/TRIM71 proteins and pluripotency

TRIM-NHL proteins are known to control the proliferation versus differentiation decision in germ- and neuronal stem cell lineages [23,25,59] and, intriguingly, *C. elegans* LIN-41 has been recently reported to control the regenerative ability of neurons [60]. While these examples highlight the importance of TRIM-NHL proteins for maintaining homeostasis in self-renewing tissues, these proteins have not previously been implicated in controlling pluripotency during development. In our study, we describe LIN-41 as a critical component of the timing mechanism controlling the oocyte reprogramming capacity. To our knowledge, this is the first example of such a regulator in cells that are ready for embryonic development, providing the initial glimpse into a pathway controlling one of the most fundamental developmental transitions. While the *in vivo* roles of the mammalian LIN-41/TRIM71 are poorly understood, the murine TRIM71 is expressed and functions in developing embryos [61,62]. TRIM71 is also preferentially expressed in embryonic stem (ES) cells [18], which are derived from pluripotent embryonic cells. In ES cells, TRIM71 represses the expression of Cdkn1, an inhibitor of the

cell cycle progression, thereby promoting proliferation [18]. While this role appears opposite to LIN-41 function in the *C. elegans* germline, TRIM71 presumably associates with many mRNAs, making additional roles likely. Intriguingly, the human LIN-41 has been recently shown to facilitate reprogramming of fibroblasts into iPSCs [28]. In this context, LIN-41, combined with several "pluripotency" transcription factors, can circumvent the requirement for c-Myc in reprogramming [28]. c-Myc facilitates reprogramming in several ways, including by inhibiting differentiation [63], and LIN-41 appears to play a similar role by repressing mRNAs encoding pro-differentiation factors [28]. Although the targets and, perhaps, the mechanisms may differ, it is striking that the *C. elegans* LIN-41 appears to fulfill an analogous function in the germline. Thus, dissecting LIN-41 targets and the mechanism are exciting objectives for the future research.

MATERIALS AND METHODS

Nematode culture, mutants, RNAi and transgenic lines

N2 animals were maintained as previously described [64] and were grown at 20°C unless stated otherwise. For alleles and transgenic lines, see Supplemental Material. For RNAi, L1 larvae (L4 for *fog-2*), grown at 25°C, were fed with bacteria expressing dsRNAs (targeting *lin-41*, *pal-1*, *fog-2* or *smg-2* from the Open Biosystem library or *lin-29* from the Ahringer library) and screened one day after the L4-to-adult molt in the same (*lin-41*, *pal-1* and *lin-29*) or in the second generation (*fog-2* and *smg-2*). A bacterial strain carrying an “empty” vector was used as a negative control (mock RNAi).

Mutagenesis and whole genome sequencing

EMS mutagenesis [64] was performed on a strain (# 1284, see Text S1) carrying the EGA-GFP (integrated at two chromosomal locations to increase GFP fluorescence). F2 animals derived from ≈ 10.000 F1s were screened. Candidate mutations were identified as previously described [65]. Each mutant was back-crossed four times against the parental strain before genome sequencing. Genomic DNAs (gDNAs) were isolated using Gentra Puregene Tissue Kit 4 g (Qiagen). DNA libraries were created from 50 ng of gDNA (Nextera DNA kit from Illumina). The sequencing data were generated using Hi Seq 2000 (Illumina).

Processing of sequence data and detection of sequence variants

Sequence reads were aligned to the May 2008 *C. elegans* assembly (obtained from http://hgdownload.soe.ucsc.edu/goldenPath/ce6/chromosomes/) using "bwa" [66]; version 0.6.1-r104) with default parameters, but only retaining single-hit alignments ("bwa samse -n 1" and selecting alignments with "X0:i:1"). The resulting alignments were converted to BAM format, sorted and indexed using "samtools" [67]; version 0.1.18). In order to quantify contamination by *Escherichia coli*, reads were similarly aligned to a collection of *E. coli* genomes (NCBI accession numbers NC_008253, NC_008563, NC_010468, NC_004431, NC_009801, NC_009800, NC_002655, NC_002695, NC_010498, NC_007946, NC_010473, NC_000913 and AC_000091), which typically resulted in less than 1% aligned reads. Sequence variants were identified using GATK [68]; version 1.5.31) indel realignment and base quality score recalibration, followed by SNP and INDEL discovery and genotyping for each individual strain using standard hard filtering parameters, resulting in a total of six to eight thousand sequence variations in each strain compared to the reference genome. Finally, the number of high quality (score >= 500) single

nucleotide substitutions of EMS-type (G/C -> A/T transitions [69], not found in other any other mutant strain or in the parent strain (typically less than 1% of the total number of variants per strain) were counted in sequential windows of 1Mb to identify regions of increased variant density.

Real-time quantitative PCR on dissected gonads

RNA was isolated from gonads dissected from one day-old (after the L4-to-adult molt) animals. cDNA was synthesized with oligo(dT) primers using the ImProm II Reverse transcription system from Promega according to manufacturer's instructions. cDNA was used for qPCR with the Absolute QPCR SYBR green ROX mix (AbGene) on an ABI PRISM 7700 system (Applied Biosystems). qPCR reactions were performed as previously described [31]. At least one primer in each pair is specific for an exon-exon junction. Human carrier RNA was added to each sample before RNA extraction, allowing normalization to hGAPDH. Standard curves for quantification were generated from a serial dilution of input cDNA for each primer pair. The amount of target present in each replicate was derived from a standard curve; an average was calculated for the triplicates. To compare total mRNA levels, the qPCR results were normalized to human GAPDH and to the wild-type values for each primer pair and fold enrichments were calculated. For primers used, see Text S1.

Immunostaining, antibodies, RNA *in situ* hybridization and microscopy

Immunostaining experiments were performed as previously described [70] with the following antibodies: PGL-1 [36] (dilution 1:1000); SPD-2 [45] ("969LA", 1:800); GFP (Roche, 1:700); phospo-Histone H3 Ser10 ("Ser10P", Millipore, 1:200); muscle myosin [71] ("5-6", 1:2.500); and UNC-120 (courtesy of Michael Krause, 1:500). Immunostainings against RME-2 [35] ("INT", dilution 1:100), GLD-1 [72] (dilution 1:5) and LIN-41 (courtesy of Helge Grosshans, "4796", 1:2.000) were performed as previously described [73] and against the Ser5P of Pol II CTD [74] ("3E8", 1:5) according to Seydoux and Dunn [33]. Immunostainings against HIM-3 [42] (courtesy of Monique Zetka, dilution 1:500) were performed as previously described [75]. Secondary antibodies used in this study: goat anti-mouse IgG alexa-488 (Molecular Probes, 1:600,), goat anti-rabbit IgG alexa-568 (Invitrogen, 1:750) and goat anti-rat IgG alexa-568 (Molecular Probes, 1:500). *In situ* hybridizations against the *vet-4* mRNA were performed as previously described [31]. Unless indicated otherwise, the gonads were dissected from 1 day-old adults. Zeiss AxioImager Z1 microscope equipped with an Axiocam MRm REV 2 CCD camera was used for capturing pictures. Images were then exported into Adobe Photoshop CS4 and processed in an identical

manner. A spinning disk multipoint confocal microscope equipped with an EM-CCD Cascade II camera (Photometrics) was used for capturing images for Figure 3D. Pictures were, then, deconvolved with the Huygens software and then processed in Imaris XP 7.1.1.

LIN-41 antibody

The affinity-purified (ELISA) rabbit anti-LIN-41 antibody ("4796") was provided by Helge Grosshans (Magdalene Rausch & Helge Grosshans, unpublished data) and created against the VKNLKLSVLISQAESLQSKQIDLQQAIQTATKLMDSSDCDEMVLRQVFEKLASCQMGNEGTEPNNNILNVLMLACQVN EDDRLKFTAPQDGILLNKARQF sequence (residues 587-686). The rabbit was raised by SDIX in Newark, DE, USA.

LIN-41 variants

The LIN-41 point mutant transgene constructs "RING" and "Y941A" were created from the wild-type LIN-41 transgenic template by site-directed mutagenesis (Stratagene QuikChange method), whereas the deletion construct "ΔNHL" was created via two-step PCR. In any case, Phusion High-Fidelity DNA Polymerase (Fermentas) was used. For primers used see Text S1.

Cloning, expression and purification of LIN-41

The filamin domain (residues 691-821) of *C. elegans* LIN-41 (isoform B of Q9U489) was cloned into pOPINF [76] using In-Fusion (Clontech Laboratories Inc). The resulting expression construct was transformed into BL21 DE3 cells and the protein expressed via auto-induction at 20°C for 20 hours. Cells were harvested, then resuspended in lysis buffer (50 mM Tris, pH 7.5, 500 mM NaCl, 20 mM imidazole, 0.2% Tween-20) and frozen at -80°C. The cell suspension was thawed and freshly supplemented with Complete EDTA-free protease inhibitors (Roche Diagnostics) and 3 U/ml Benzonase (Sigma) before passing through an Avestin EmulsiFlex-C3 cell disruptor. The clarified lysate was incubated with NiNTA affinity resin (Qiagen) in batch mode and the bound protein eluted in 50 mM Tris, pH 7.5, 500 mM NaCl, 125 mM imidazole. The protein was fractionated on a Superdex 75 HiLoad 16/60 (GE Healthcare) gel filtration column in GF buffer (20 mM Tris, pH 7.5, 200 mM NaCl, 2 mM TCEP and 0.02% NaN_3). The single peak fraction was pooled and digested overnight at 4°C with 3C protease to remove the N-terminal histidine tag. The released protein tag and 3C protease were removed by a second nickel-

affinity step and the untagged filamin domain was further purified over a Superdex 75 column in GF buffer and concentrated to 7.5 mg/ml.

Crystallization, data collection and structure solution

All crystallization experiments were performed at 20°C using the sitting-drop vapour diffusion method via a Phoenix robot (Art Robbins) dispensing 100 nl drops. Removal of the N-terminal histidine tag from the filamin domain was needed to obtain crystals. The untagged filamin domain readily crystallized in many conditions. Crystals grown in 1.1 M sodium malonate, 0.1 M HEPES, pH 7.0, 0.5% v/v Jeffamine ED-2001, were harvested and cryoprotected in mother liquor containing 25% ethylene glycol. These crystals diffracted to 1.68 Å resolution at the SLS PX-III beamline and belonged to space group $C222_1$ with one molecule per asymmetric unit. Diffraction data were integrated and scaled using XDS [77] and the structure was solved by the molecular replacement method using PHASER [78]. Phases from this solution were calculated and used for automatic model building with BUCCANEER [79]. The LIN-41 filamin structure was further improved by the crystallographic simulated annealing routine followed by individual B-factor refinement in PHENIX [80] and several rounds of manual rebuilding in COOT [81] and refinement in BUSTER [82]. The final structure was validated using COOT. Structural images for figures were prepared with PyMOL (http://pymol.sourceforge.net/). Atomic coordinates and structure factors for the LIN-41 filamin domain have been deposited in the PDB with entry code 4UMG.

Homology modeling

Amino acid sequences of the *C. elegans* LIN-41 (Uniprot Q9U489, 830-1147) and *Homo sapiens* TRIM71 (Uniprot Q2Q1W2, 591-868) NHL domains were submitted to the HHPRED server for homology detection and structure prediction [83]. The structure of the *D. melanogaster* Brat NHL domain (PDB 1Q7F) was the top hit in both searches resulting in very high scores for the LIN-41 NHL domain (Score=241.22, E-value=1e-33, 28% sequence identity) and the TRIM71 NHL domain (score=22.54, E-value=4.5e-35, 31% identity). The top alignments were edited for minimal local corrections and sent to the HHPRED MODELLER pipeline for modeling. Loops lacking template information for both the *C. elegans* and the human NHL domain models were removed in the final models. Structural figures were prepared using PyMOL (www.pymol.org).

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FIGURE LEGENDS

Figure 1. LIN-41 prevents activation of embryonic transcription in the germline.

A. Summary of a genetic screen to identify mutants inducing EGA in the adult germline. In wild-type, the EGA-GFP reporter (green, marking embryonic transcription) is expressed in embryos. In mutants, this reporter is abnormally expressed in the germline. Asterisks here and in the subsequent figures mark the distal end of the gonad. **B.** Fluorescent micrographs of live animals expressing EGA-GFP. The gonads are outlined with a continuous line, the embryos and animals with dashed lines. Arrows point to selected embryos, the older of which express EGA-GFP. The *lin-41(rrr3)* mutants express EGA-GFP abnormally in the proximal gonad (boxed in red). This phenotype was observed in all examined animals (n > 100). Scale bar: 25 µm. **C.** LIN-41 protein domains and their putative functions. Mutations identified in this study are indicated. **D.** *In situ* hybridization against an endogenous EGA transcript, *vet-4.* Shown are light micrographs of gonads and wild-type embryos (at the indicated stages), which were hybridized with antisense (AS) or sense (S) probes for the *vet-4* mRNA. In contrast to the wild-type gonads, *vet-4* mRNA was detected in the proximal region of all *lin-41(rrr3)* gonads (boxed in red; n = 20). Scale bar: 20 µm. **E.** Detection of additional EGA transcripts by RT-qPCR. "Early embryonic": mRNAs normally expressed in the early embryo following EGA. Each bar represents the mean of three independent biological replicates, the error bars represent the standard error of the mean (SEM) and the significance of the differences has been calculated with the Student's t-test (symbols: "*", $p < 0.05$; "n.s.", not significant).

Figure 2. Cells in the proximal regions of *lin-41* gonads lose germline characteristics and differentiate into somatic cells, forming a teratoma.

A. Fluorescent maximum intensity projections of gonads immunostained for the transcription-activating phosphorylation of Ser5 within the Pol II CTD (Ser5P). This phosphorylation was absent in the most-proximal wild-type gonads, but was present in all corresponding *lin-41(rrr3)* gonads (n > 40). Encircled in red are nuclei containing low or no Ser5P. The corresponding DAPI-stained nuclei, from the boxed areas, are on the right. The proximal-most *lin-41* gonad contains sperm (lightly colored in A and C), which explains the lack of Ser5P in this region. Scale bar: 10 µm. **B.** Fluorescent maximum intensity projections of gonads immunostained for an oocyte-expressed protein, the yolk receptor RME-2. In wild-type gonads, RME-2 is expressed in developing oocytes. In *lin-41(rrr3)* gonads, RME-2 is expressed in oocyte-like cells but is absent from the most proximal cells (boxed). Scale bar: 25 µm. **C.** Fluorescent maximum intensity projections of gonads immunostained for a germline-specific protein, PGL-1. PGL-1 (concentrated in RNA/protein granules) is present throughout the wild-type gonad but is eliminated in

the proximal *lin-41(rrr3)* gonad (all gonads, n = 25). The corresponding DAPI-stained nuclei from the boxed areas are on the right. Scale bar: 10 µm. **D.** Detection of somatic cell-specific transcripts by RT-qPCR. "Somatic lineage-specific" indicates mRNAs expressed in somatic cell lineages: muscle (*hlh-1*, *unc-120)* or pharynx/gut (*end-1*, *end-3*, *elt-2, pha-4* and *tbx-2*). "Hox" genes direct various aspects of somatic development. Each bar represents the mean of three independent biological replicates, the error bars represent the SEM and the significance of the differences has been calculated with the Student's t-test ("*", $p < 0.05$; "**", $p < 0.01$; "n.s.", not significant). **E.** Fluorescent maximum intensity projections of gonads immunostained for the muscle-lineage marker UNC-120 and for a neuronal GFP reporter (nGFP). Arrows point to UNC-120-containing cells of the somatic gonad. In contrast to wild-type, UNC-120 and nGFP-expressing cells (boxed) are present within the *lin-41(rrr3)* germline. The inset shows nGFP-expressing cells from a different *lin-41(rrr3*) gonad, which extend long, neuronal-like processes (arrowhead). Scale bar: 50 µm.

Figure 3. LIN-41 inhibits the transition from meiosis to mitosis in developing oocytes.

A. Time-course of EGA, mitotic chromosome condensation, spindle formation and somatic-like differentiation in *lin-41(rrr3)* gonads. The numbers indicate the fractions of *lin-41(rrr3)* gonads expressing EGA-mCherry (here the *vet-4* promoter drives mCherry-tagged H2B) or assembling mitotic spindles (visualized with GFP-tagged □-tubulin; TBB-2-GFP), which was observed in live animals at the indicated time points. Additionally, these animals were immunostained for a mitotic marker (Ser10P) or UNC-120. At least 30 gonads were examined per each time-point/marker. **B.** Fluorescent maximum intensity projections of selected regions (boxed in red on the schematic gonads) of wild-type and *lin-41(rrr3)* gonads, immunostained for Ser10P and microtubule spindle (TBB-2-GFP), also stained by DAPI. Scale bars: 10 µm. **C.** Fluorescent maximum intensity projections of selected regions of wild-type and *lin-41(rrr3)* central-proximal gonads, immunostained for the meiotic marker HIM-3 and also stained by DAPI. Scale bars: 25 µm. **D.** Confocal images of maximum intensity projections of selected cells in the proximal *lin-41(rrr3)* gonad stained by DAPI, immunostained for the centrosomal component SPD-2 and for TBB-2-GFP, one day after the L4-to-adult molt. In contrast to wild-type gonads (not shown), cells in the proximal *lin-41* gonads contained duplicated centrosomes (red), facilitating the assembly of microtubule spindles (green). Number of observed *lin-41* cells forming a spindle with duplicated centrosomes: 60/60. Scale bar: 10 µm.

Figure 4. LIN-41 controls the germline-to-soma transition autonomously in the germline.

A. DIC micrographs of live animals, either wild-type (upper panels) or *lin-41(rrr3)* (lower panels), carrying a GFP-LIN-41 rescuing transgene driven by a heat-shock promoter (*hsp-16.41*). Control animals grown at 20°C are shown on the left and animals grown at 25°C (allowing leaky expression form the heat-shock promoter) on the right. Scale bars: 50 µm. **B.** Left panel: a DIC micrograph of a live *lin-41(rrr3)* animal grown at 25°C. The gonad is outlined with a dashed line. Note the absence of oocytes and the presence of a proximal germline tumor. Scale bar: 50 µm. Right panel: fluorescent maximum intensity projections of a small area from a proximal gonad immunostained for the muscle-lineage marker UNC-120, indicating teratoma formation in *lin-41(rrr3)* animals grown at 25°C. Scale bar: 10 µm. **C.** Gonads of the indicated genotypes immunostained for LIN-41. Scale bar: 50 µm.

Figure 5. LIN-41 does not function as an E3 ubiquitin ligase.

A. Sequence alignment of the mammalian TRIM71/LIN-41 RING domain with those from *Caenorhabditis* species and different known E3 ubiquitin ligases. Asterisks indicate the position of conserved cysteines (*C. elegans*: C114, C117, C130, C151, C154), which have been mutated to serines (S) to disrupt the domain (LIN-41^{RING}). A highly conserved proline residue (P, highlighted in yellow), which is critical for canonical E3-E2 interactions, is absent in the nematode LIN-41 proteins. **B.** Fluorescent micrographs of live animals expressing wild-type (LIN-41^{WT}) and mutated (LIN-41^{RING}) GFP-tagged LIN-41 in the *lin-41(rrr3)* mutant background. The gonads are outlined with a dashed line. No differences in the distribution or in the levels of the two proteins have been observed (n > 50). Scale bar: 20 µm.

Figure 6. The filamin domain of LIN-41 is not essential for the germline function.

A. Left: fluorescent micrographs of gonads of the indicated genotypes immunostained for LIN-41. Right: corresponding maximum intensity projections of DAPI staining of the proximal-most region of the gonads shown on the left. Scale bars: 25 µm. **B.** Amino acid sequences of the filamin domain for wild-type and *lin-41(ma104)* mutant (derived from a cDNA sequence). **C.** Crystal structure of the LIN-41 filamin domain. The domain is presented as a cartoon model in rainbow colors from blue (N-terminus) to red (C-terminus) with a transparent surface. The position and sequence of the 16-residue insert present in the *lin-41*(*ma104)* allele in the second β-strand is highlighted. The disordered sequence stretch 730-757 which is not included in the model is displayed as grey dots.

Figure 7. LIN-41 may control the germline-to-soma transition independently from its role in mRNA regulation.

A. Fluorescence micrographs of *lin-41(rrr4)* gonads stained for LIN-41. Left: suppressing nonsense-mediated mRNA decay (by *smg-2 RNAi*) restores the expression of LIN-41^{rrr4}. Right: by maximum intensity projections of DAPI staining, LIN-41^{rrr4} does not rescue the oocyte defects observed in LIN-41-depleted gonads. In contrast to the wild-type gonad containing oocytes in the proximal-most region (I), the gonad expressing LIN-41^{rrr4} (II) (n = 15) accumulates smaller nuclei, which are similar to those in the LIN-41-depleted gonad (III). Scale bars: 50 µm. **B.** Top: a schematic view of the LIN-41 NHL domain and its six β-propellers marked in red (I-VI). Previously identified mutations [29] and our point mutant "LIN-41^{Y941A}" are indicated. Bottom left: a homology model of the LIN-41 NHL domain viewed from the electropositive side. The NHL propeller blades are numbered (I-VI) and N-/C-termini are indicated. The residue Y941 is shown in orange atom colors. Known substitution mutations [29] are displayed as sticks in cyan (atom colors). Loops that could not be modeled due to lack of homology are shown as dotted lines. Bottom right: surface representation of the LIN-41 NHL domain homology model in two orientations (rotated by 180° along a horizontal axis). Fully conserved surface-exposed residues are marked in red (see alignment in Figure S8). Y941 (in orange) is in the center of the highly conserved surface patch on the electropositive side of the NHL domain. **C.** Left: gonads expressing the indicated GFP-tagged LIN-41 variants in otherwise *lin-41(rrr3)* gonads. Right: by maximum intensity projections of DAPI staining, LIN-41$^{\Delta NHL}$ did not rescue the oocyte defects, as evident by the accumulation of smaller nuclei similar to those in the LIN-41-depleted gonad. In contrast, gonads expressing LIN-41^{Y941A} contained overall normal oocytes and LIN-41^{Y941A} rescued the sterility of *lin-41(rrr3)* animals. At least 50 gonads per strain were examined. Scale bars: 50 µm. **D.** *In situ* hybridization against an endogenous EGA transcript, *vet-4.* Shown are light micrographs of gonads and embryos (at the indicated cell stages, "c.s."), which were hybridized with antisense (AS) or sense (S) probes for the *vet-4* mRNA. Similarly to the gonads expressing the rescuing LIN-41^{WT}, *vet-4* mRNA was absent from the gonads expressing LIN-41^{Y941A}. Scale bar: 50 µm.

SUPPORTING INFORMATION

SUPPLEMENTAL TEXT

Text S1. Supplemental materials and methods.

Complete lists of alleles and transgenic lines, RT-qPCR primers, primers used to create the mutated transgenes for *lin-41* and the accession numbers for proteins used in alignments in this study are provided.

SUPPLEMENTAL FIGURES

Figure S1. Mutations in the *lin-41* gene cause precocious onset of embryonic transcription in the germline.

A. Following EMS-induced mutagenesis and outcrossing of two similar mutants (*rrr3* and *rrr4*) against the wild-type parental strain, whole genome sequencing uncovered sequence variants clustering on chromosome I. Numbers indicate chromosomes and "M" mitochondrial DNA. Genes containing EMS-type mutations and present within the chromosomal regions highlighted in yellow are listed in the table below. **B.** A summary of candidate mutations from the chromosomal regions highlighted above. The only gene mutated in both strains, *lin-41*, is highlighted in red. **C.** Fluorescent micrographs of live wild-type animals expressing EGA-GFP subjected to either mock or *lin-41 RNAi*. The gonads are outlined with a continuous line and the embryos and animals with dashed lines. Consistent with the phenotype of *lin-41(rrr3)* and *lin-41(rrr4)* mutants, *lin-41(RNAi)* animals (examined at 42 hours post-L1 stage) expressed EGA-GFP in the proximal gonad (boxed in red). This phenotype was fully penetrant (n = 50). Scale bar: 50 µm. **D.** DIC (upper panel) and fluorescent (lower panel) micrographs of live *lin-41(rrr3)* animals expressing the FLAG- and GFP-tagged LIN-41 rescuing construct. The gonads and the embryos are outlined with white dashed lines, the spermatheca with red dashed lines (note the absence of LIN-41 from sperm) and the animals with continuous lines. Early embryonic stages (c.s.) are indicated. Scale bar: 50 µm.

Figure S2. GLD-1 expression is not altered in *lin-41* mutant gonads.

Fluorescent micrographs of wild-type and *lin-41(rrr3)* gonads immunostained for GLD-1. Scale bar = 50 µm.

Figure S3. Differentiation into muscles in *lin-41* teratomas.

Fluorescent maximum intensity projections of proximal gonads immunostained for muscle myosin. In contrast to wild-type gonads, which contain myosin only in the somatic sheath cells that envelop the germline, clusters of myosin-expressing cells (arrows) are present within the proximal region of the *lin-41(rrr3)* gonads. Scale bars: 20 µm.

Figure S4. PAL-1 is expressed in both wild-type and *lin-41* gonads.

A. Fluorescent maximum intensity projections of gonads expressing GFP-tagged PAL-1, dissected from 0.5 day-old animals subjected to either mock or *lin-41 RNAi*. Scale bar: 50 µm. **B.** Upper panel: a simplified view of PAL-1-dependent transcriptional cascade. Lower panels: left: fluorescent maximum intensity projections of *lin-41(rrr3)* gonads stained for UNC-120. Scale bar = 25 µm. Right: the corresponding quantification. The numbers of UNC-120 expressing cells per gonad were significantly reduced upon *pal-1 RNAi* (number of analyzed gonads = 37) compared to mock RNAi (number of analyzed gonads = 20) ($p < 0.001$). The error bars represent the SEM.

Figure S5. Centrosome duplication in *lin-41* proximal gonads does not represent an aberrant spermatogenesis.

Fluorescent maximum intensity projections of selected gonadal cells immunostained for the centrosomal component SPD-2 (red) and stained by DAPI (green). L4 animals had been subjected to *fog-2 RNAi* and their progeny were used for the experiment. Only animals fully sperm-depleted were analyzed. Upper panels: selected cells from wild-type gonads. Lower panels: selected cells from *lin-41(rrr3)* gonads. Left panels: cells from the distal region of the gonad, where mitotic cells reside and normally exhibit centrosome duplication (arrowheads point to examples of duplicated centrosomes in both wild-type and mutant gonads). Right panels: cells from the proximal region of the gonad. Feminized wild-type proximal gonads contain oocytes where centrosomes have been eliminated; feminized *lin-41(rrr3)* proximal gonads contain centrosomes that have not been eliminated and some of them display duplication (arrowheads). Scale bars = 10 µm.

Figure S6. LIN-41 is down-regulated upon ovulation and is absent from early embryos.

Fluorescent micrographs of wild-type gonads and early embryos immunostained for LIN-41 (upper panel) and DAPI (lower panel). Shown are maximum intensity projections. The gonads and the embryos are outlined with dashed lines. Scale bar: 50 µm.

Figure S7. The absence of LIN-29 does not interfere with proximal tumor formation of *lin-41* gonads.
DIC micrographs of a live *lin-41(rrr3); lin-29(n546)* double mutant worm; the gonad is outlined with white-dashed lines and the proximal tumor is in red. An asterisk marks the distal end of the gonad and an arrowhead the vulval defects. Scale bar: 50 µm.

Figure S8. Clustal Omega multiple sequence alignment of LIN-41 NHL domain orthologous sequences.
The alignment was calculated using the EBI ClustalO server (http://www.ebi.ac.uk/Tools/msa/clustalo/). Fully conserved residues (similarity groups enabled) are shaded in black and mapped onto the surface of the *C. elegans* LIN-41 NHL domain homology model in Figure 7B.

Figure S9. Homology models of the NHL domains of LIN-41 and TRIM71.
A. Superpositions of the NHL domain homology models of *C. elegans* LIN-41 (left, displayed in grey as in Figure 7B) and *Homo sapiens* TRIM71 (right, in dark purple) onto the X-ray structure of *D. melanogaster* Brat NHL domain (PDB 1Q7F, in blue). Brat residues important for binding of the Pumilio Puf domain are shown as green sticks in atom colors [52]. Residues of the human TRIM71 NHL domain involved in mRNA repression are displayed as sticks in magenta [19]. **B**-**C.** HHPRED alignments between *C. elegans* LIN-41 (B) and *H. sapiens* TRIM71 (C) NHL domain sequences with the sequence of the *D. melanogaster* Brat NHL domain of known structure (PDB 1Q7F). Individual alignments were used to calculate the *C. elegans* and *H. sapiens* NHL domain homology models, respectively. Residues highlighted in the structural superpositions in (A) are indicated in the alignments.

SUPPLEMENTAL TABLE

Table S1. Data collection and refinement statistics for the LIN-41 filamin domain.

Diffraction data collection statistics for a crystal of the LIN-41 filamin domain are presented in the upper part (Data collection), while statistics for the final structural model and its fit against the experimental data are presented below (Refinement).

SUPPLEMENTAL MATERIALS AND METHODS

Alleles and transgenic lines

CGC or lab number	Genotype
1284	*rrrSi199[Pvet-4::NLS:gfp:gfp::vet-4 3'UTR; unc-119(+)] (II); rrrSi198[Pvet-4::NLS:gfp:gfp::vet-4 3'UTR; unc-119(+)] (IV)*
1180	*lin-41(rrr3)/unc-29(e1072) lin-11(n1281) (I); rrrSi199 (II); rrrSi198 (IV)*
MT7897	*lin-41(n2914)/unc-29(e1072) lin-11(n1281) (I)*
OH441	*otIs45[unc-119::gfp] (V)*
1283	*lin-41(n2914) (I); otIs45 (V)*
1252	*ojIs1[tbb-2::gfp], derived from TY3558*
1282	*lin-41(rrr3) (I); ojIs1*
1285	*lin-41(rrr4) (I); rrrSi199 (II); rrrSi198 (IV)*
1414	*lin-41(n2914) (I); rrrSi308[Plin-41::lin-41(*C114S, C117S, C130S, C151S, C154S*)::lin-41 3'UTR; unc-119(+)] (II)*
1515	*lin-41(rrr3)/hT2[qIs48] (I;III); lin-29(n546)/mnC1[dpy-10(e128) unc-52(e444) nIs190] (II)*
RW10993	*unc-119(ed3) (III); itIs37[Ppie-1::mCherry:h2b::pie-1 3'UTR; unc-119(+)]; stIs10116[Phis-72::his-24:mCherry::let-858 3'UTR; unc-119(+)]; wgIs94[Ppal-1::TY1:eGFP:3xFLAG(P000006_G02)]*
1328	*lin-41(rrr3) (I); rrrSi197[Pvet-4::NLS:mCherry:h2b::vet-4 3'UTR; unc-119(+)] (II) ; ojIs1*
1320	*lin-41(rrr3) (I); [Plin-41::flag:gfp:lin-41::lin-41 3'UTR; unc-119(+)] (II)*
1334	*lin-41(rrr3) (I); rrrSi304[Plin-41::flag:gfp:ΔNHL::lin-41 3'UTR; unc-119(+)] (II)*
1344	*lin-41(rrr3) (I); rrrSi309[Plin-41::flag:gfp:Y941A::lin-41 3'UTR; unc-119(+)] (II)*

1412	*lin-41(rrr3)/+ (I); rrrSi298[Phsp-16.41::flag:gfp:lin-41::lin-41 3'UTR; unc-119(+)] (II)*
1468	*lin-41(ma104) (I); rrrSi199[Pvet-4::NLS:gfp:gfp::vet-4 3'UTR; unc-119(+)] (II)*

Real-time quantitative PCR on dissected gonads

Primers used:

act-1 FW	CTATGTTCCAGCCATCCTTCTTGG
act-1 RV	TGATCTTGATCTTCATGGTTGATGG
tbb-2 FW	GCTCATTCTCGGTTGTACCA
tbb-2 RV	TGGTGAGGGATACAAGATGG
vet-1 FW	AAAGAACTGAAACTATGTTTGCTG
vet-1 RV	CTCTCGTCGTGTTTTCTGATG
vet-4 FW	AAGGATTTCACTGCTTGCTC
vet-4 RV	CGTCGTTTTCGATTTCTCCG
vet-6 FW	GTGCGAGACAAGAATGTAATCC
vet-6 RV	TTCTTGAACTCTTGGAACACAG
pes-10 FW	GCGATGATTTCATGATTTCCTG
pes-10 RV	AATTTCGTAGTCAATCTGCTCC
hlh-1 FW	ACGATTATGTGACTTCCTCTC
hlh-1 RV	GATGATCTCTATCGTCGTCC
unc-120 FW	GGGTATTATGAAGAAGGCATTCG
unc-120 RV	TGCATATGTGTAGACATGACCA
end-1 FW	GGGCAATACTTTGTTCAATCG
end-1 RV	GGATACTGTTGTGAGTAGCA
end-3 FW	GCCTATTAATGACCTCCAGC
end-3 RV	CCCGTCAATTGGTATCTCTG

elt-2 FW	AGTAAACGGAGGAATGATGTG
elt-2 RV	CTGCTCTGAAGGTATTTCCA
pha-4 FW	CCAGAATTTCCTGAACAACAC
pha-4 RV	GTTGGTGGAGCTGTAAAGAG
tbx-2 FW	AAGTGGAAGACGGATATTCC
tbx-2 RV	TTGTAACGGTGTTCATCAGC
mab-5 FW	TTCATCAAATCCATTCGCCT
mab-5 RV	CATGGAAATACTGGTTGCGA
ceh-13 FW	CAGCATAACACATACAAGTGG
ceh-13 RV	GAAGTTGGTTCGATTTGTTCC
php-3 FW	TTATCAAGGACACAAGCGGA
php-3 RV	ATTGACATACCACTGCTCGT
hGAPDH FW	GGAGTCAACGGATTTGGTC
hGAPDH RV	AAACCATGTAGTTGAGGTC

Mutating LIN-41

The following primer pairs were used to introduce the point mutations:

Fw_C130S GAGTGCTGCCGTTCGTTTCAGCGCACAAGTGGGGTTTC

Rv_C130S GAAACCCCACTTGTGCGCTGAAACGAACGGCAGCACTC

Fw_C114S_C117S GCAAGACTCCTTTCGGTCCTCAGTCTCCTCCAAGAGCTCGAC

Rv_C114S_C117S GTCGAGCTCTTGGAGGAGACTGAGGACCGAAAGGAGTCTTGC

Fw_C151S_C154S GAATTTTAGCTTCCAAGCTGTCAGGTGCCGTATCGACAGCC

Rv_C151S_C154S GGCTGTCGATACGGCACCTGACAGCTTGGAAGCTAAAATTC

Fw_Y941A GTTGGCTACTTCAACGCTCCATGGGGAGTTGC

Rv_Y941A GCACATCCCCATGGAGCGTTGAAGTAGCCAAC

The following primer pair was used to create the NHL deletion mutant:

Fw_ΔNHL GATCCGGTGACGGAGAATAGACACTTTCTTCTTGC

Rv_ΔNHL GCAAGAAGAAAGTGTCTATTCTCCGTCACCGGATC

Accession numbers of proteins used in alignments

Q3B891 (UniProt). Protein: BRCA1. Species: *Homo sapiens*.

Q99496 (UniProt). Protein: RING2. Species: *Homo sapiens*.

Q13489 (UniProt). Protein: BIRC3. Species: *Homo sapiens*.

Q8WY64 (UniProt). Protein: MYLIP. Species: *Homo sapiens*.

P22681 (UniProt). Protein: CBL. Species: *Homo sapiens*.

E3MFJ2 (UniProt). Protein: LIN-41. Species: *Caenorhabditis remanei*.

G0MLY8 (UniProt). Protein: LIN-41. Species: *Caenorhabditis brenneri*.

Q9U489 (UniProt). Protein: LIN-41. Species: *Caenorhabditis elegans*.

Q2Q1W2 (UniProt). Protein: TRIM71. Species: *Homo sapiens*.

E1BJS7 (UniProt). Protein: TRIM71. Species: *Bos taurus*.

D3ZVM4 (UniProt). Protein: TRIM71. Species: *Rattus norvegicus*.

Q1PRL4 (UniProt). Protein: TRIM71. Species: *Gallus gallus*.

F6QEU4 (UniProt). Protein: TRIM71. Species: *Xenopus tropicalis*.

E7FAM5 (UniProt). Protein: TRIM71. Species: *Danio rerio*.

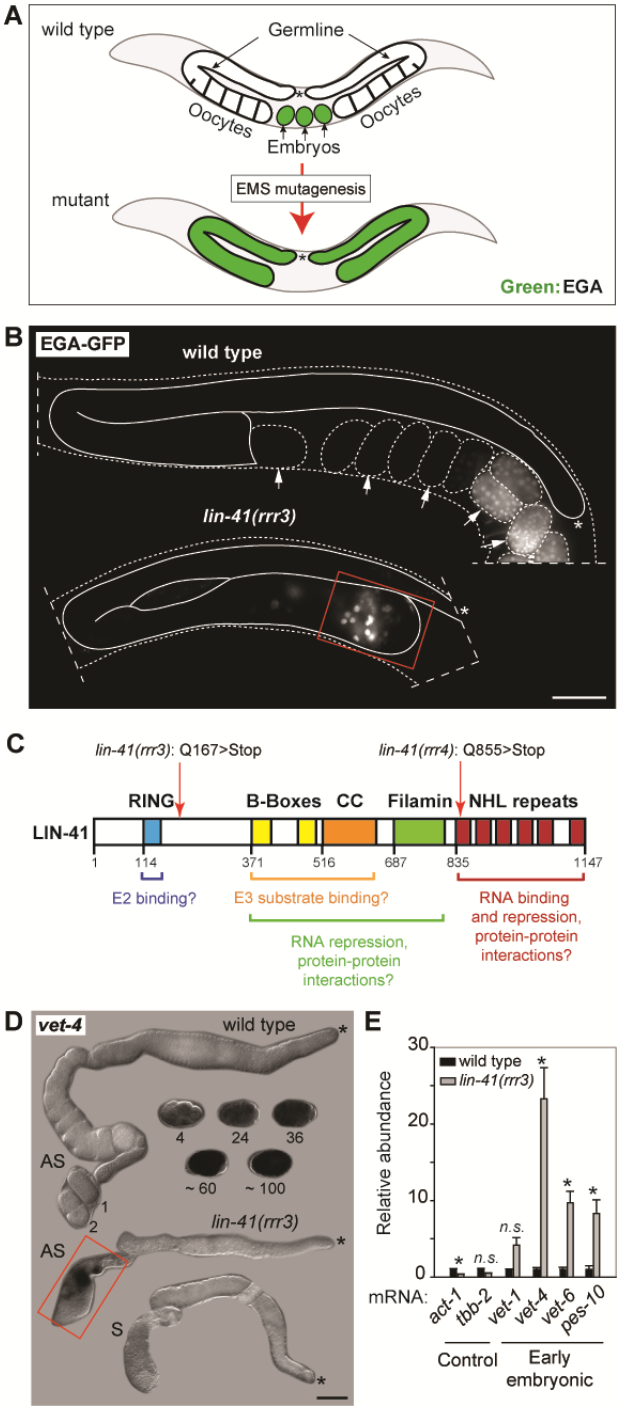
Q9V4M2 (UniProt). Protein: WECH. Species: *Drosophila melanogaster*.

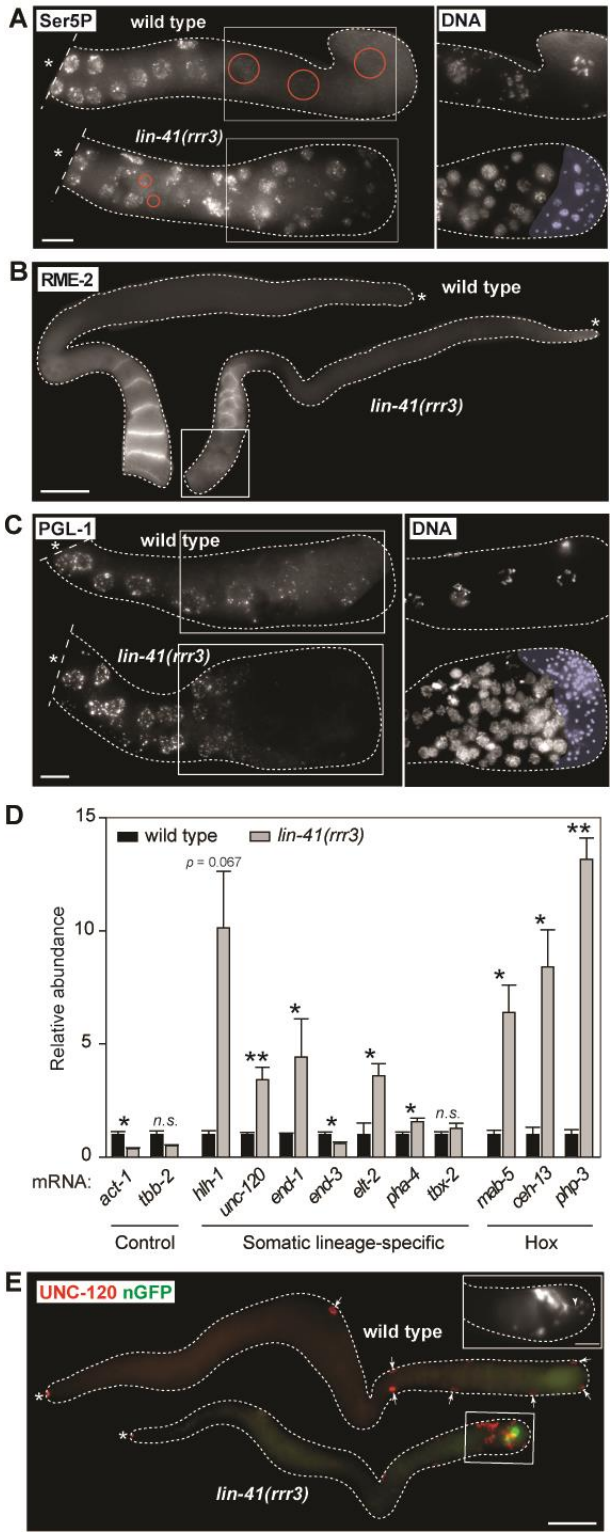
1Q7F (PDB ID). Protein: Brat. Species: *Drosophila melanogaster*.

Table S1. Data collection and refinement statistics for the LIN-41 filamin domain.

Data collection	
Space group	$C222_1$
Cell const. *a, b, c* [Å]	44.78, 52.11, 101.31
Wavelength [Å]	1.000
Resolution range [Å][a]	50.0-1.68 (1.72-1.68)
Unique reflections	13883
Completeness [%][a]	99.9 (100.0)
Multiplicity	7.0
R_{sym} [%][a]	4.5 (87.8)
$I/\sigma(I)$[a]	20.8 (1.9)
CC(1/2) [%][a]	99.9 (69.3)
Refinement	
Resolution range [Å]	50.0-1.68
Reflections (all)	13854
Reflections (test set)	693 (5.0 %)
R_{crys} [%]	21.9
R_{free} [%]	24.4
RMSDs	
Bond lengths [Å]	0.01
Bond angles [°]	1.11
Ramachandran plot [%]	
Allowed	100
Outliers	0

[a]Values in parentheses refer to the highest resolution shell.


A
wild type
Germline
Oocytes
Oocytes
Embryos
EMS mutagenesis
mutant
Green: EGA
B
EGA-GFP
wild type
lin-41(rrr3)
C
lin-41(rrr3): Q167>Stop
lin-41(rrr4): Q855>Stop
RING
B-Boxes
CC
Filamin
NHL repeats
LIN-41
1
114
371
516
687
835
1147
E2 binding?
E3 substrate binding?
RNA binding and repression, protein-protein interactions?
RNA repression, protein-protein interactions?
D
vet-4
wild type
4
24
36
~ 60
~ 100
AS
1
2
lin-41(rrr3)
AS
S
E
Relative abundance
wild type
lin-41(rrr3)
n.s.
n.s.
mRNA:
act-1
tbb-2
vet-1
vet-4
vet-6
pes-10
Control
Early embryonic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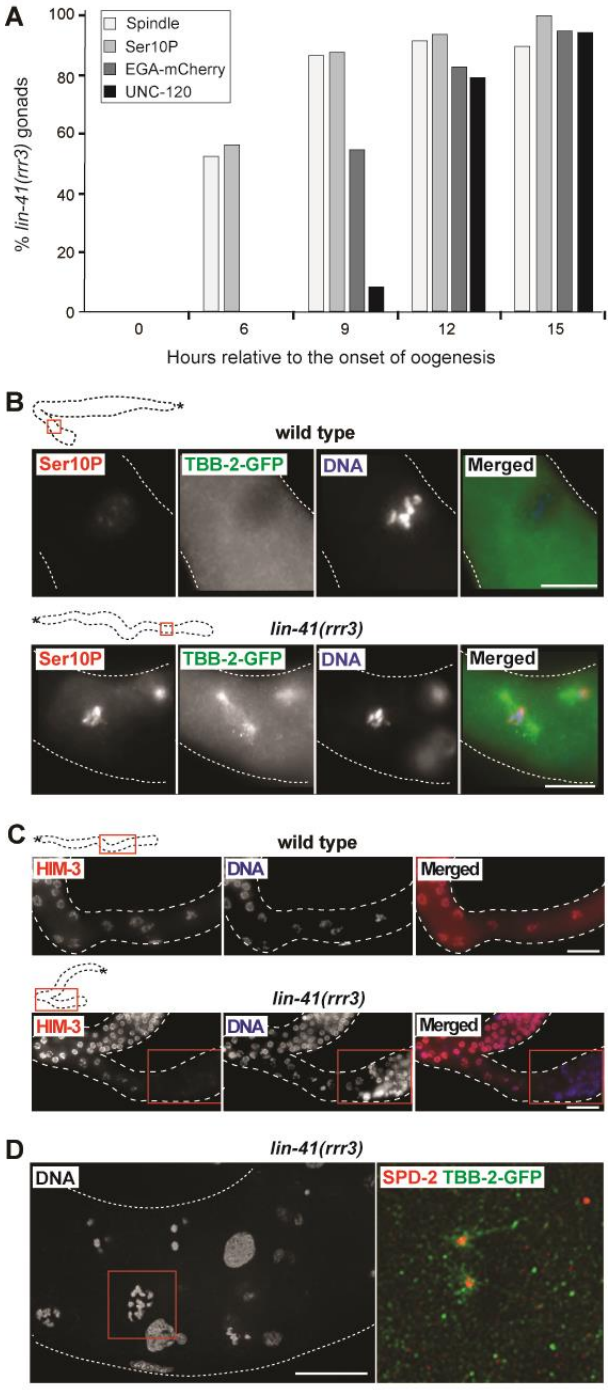
Ser5P
wild type
DNA
lin-41(rrr3)
B
RME-2
wild type
lin-41(rrr3)
C
PGL-1
wild type
DNA
lin-41(rrr3)
D
wild type
lin-41(rrr3)
p = 0.067
Relative abundance
15
10
5
0
n.s.
n.s.
mRNA:
act-1
tbb-2
hlh-1
unc-120
end-1
end-3
elt-2
pha-4
tbx-2
mab-5
ceh-13
php-3
Control
Somatic lineage-specific
Hox
E
UNC-120 nGFP
wild type
lin-41(rrr3)

Tocchini_Fig 3



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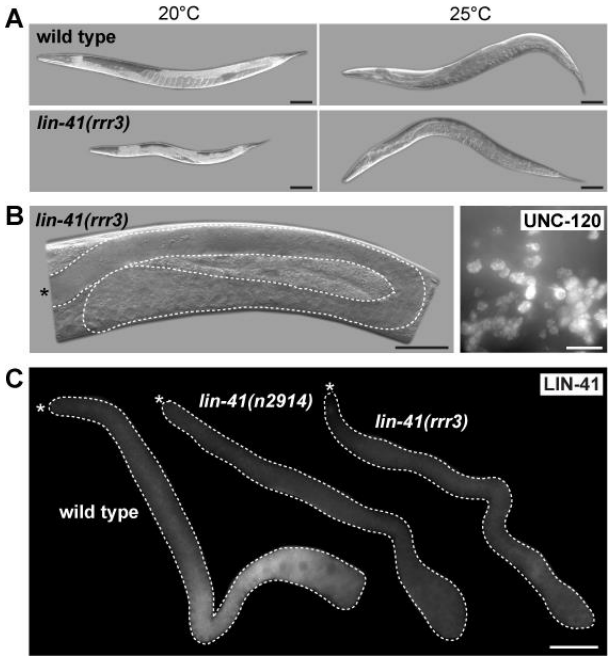

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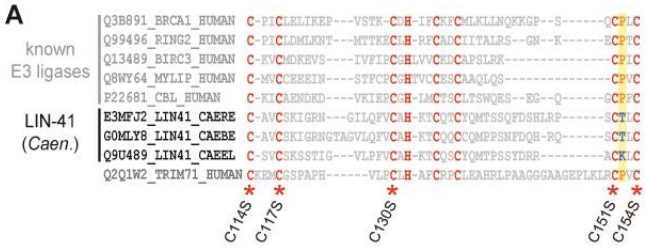

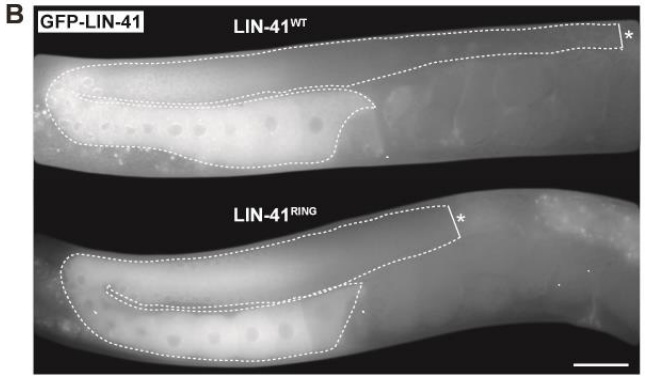

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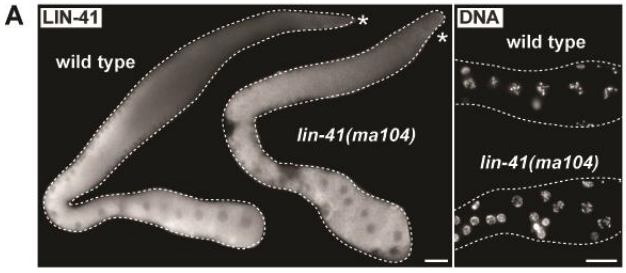

B

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Filamin_ma104   :  AKNSSIVGDSFKKAIRERQTVIYSAGQKDIHFWFFPALYVQLRDACGDLLSSSIAATQPT
                   **********************                 **********************

Filamin_WT      :  SQALLPHQEPHSHLEQAMPTSDVQAFVISPDGSTVEVTMTPRENGIVALSYYPSIEGSYT  798
Filamin_ma104   :  SQALLPHQEPHSHLEQAMPTSDVQAFVISPDGSTVEVTMTPRENGIVALSYYPSIEGSYT
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Filamin_WT      :  LNILVKGTPISGCPT  813
Filamin_ma104   :  LNILVKGTPISGCPT
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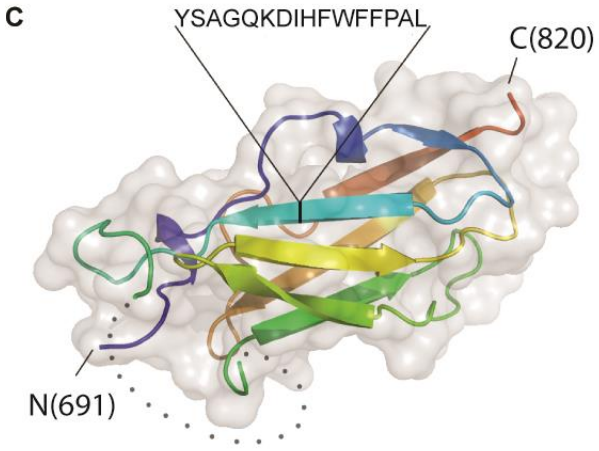
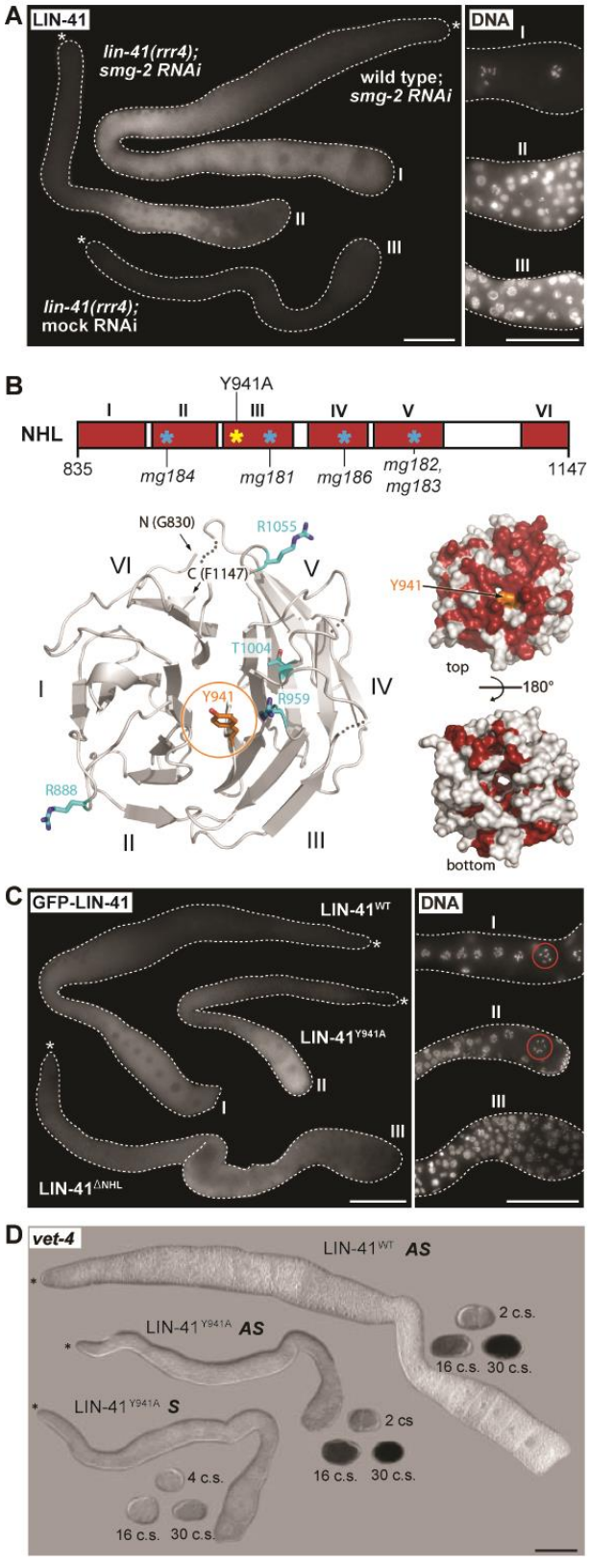
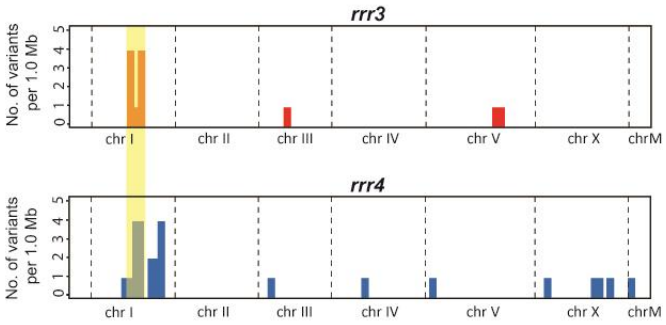



A
LIN-41
lin-41(rrr4); smg-2 RNAi
wild type; smg-2 RNAi
lin-41(rrr4); mock RNAi
DNA
I
II
III
B
Y941A
NHL
I II III IV V VI
835
mg184
mg181
mg186
mg182, mg183
1147
N (G830)
C (F1147)
R1055
T1004
R959
R888
Y941
top
180°
bottom
C
GFP-LIN-41
LIN-41WT
LIN-41Y941A
LIN-41ΔNHL
DNA
D
vet-4
LIN-41WT AS
LIN-41Y941A AS
LIN-41Y941A S
2 c.s.
16 c.s.
30 c.s.
2 cs
4 c.s.

Tocchini_Fig S1

A



B

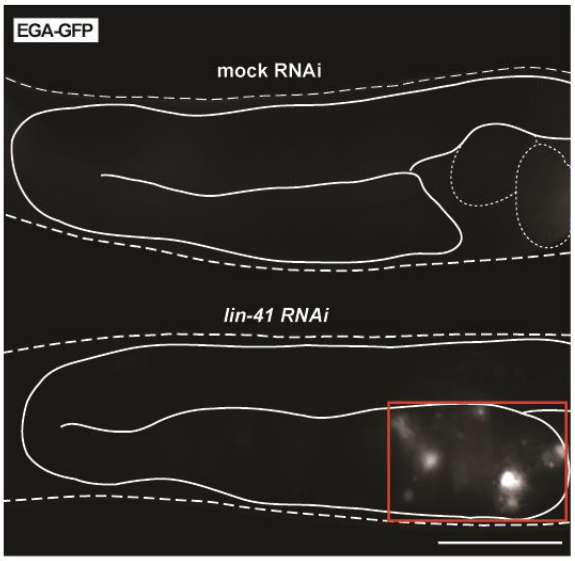
rrr3

Overlapping Gene	Chr.	Genomic position	Molecular change	Type of mutation	Location
F30F8.1	I	7833482	Substitution	Silent	Exon
H05L14.2	I	7989887	Substitution	Missense	Exon
lin-41	I	9341257	Substitution	Nonsense	Exon
C17E4.1	I	9408529	Substitution	Missense	Exon

rrr4

Overlapping Gene	Chr.	Genomic position	Molecular change	Type of mutation	Location
twk-30	I	8809016	Substitution	Missense	Exon
hsp-70	I	9321629	Substitution	Nonsense	Exon
lin-41	I	9336930	Substitution	Nonsense	Exon

C



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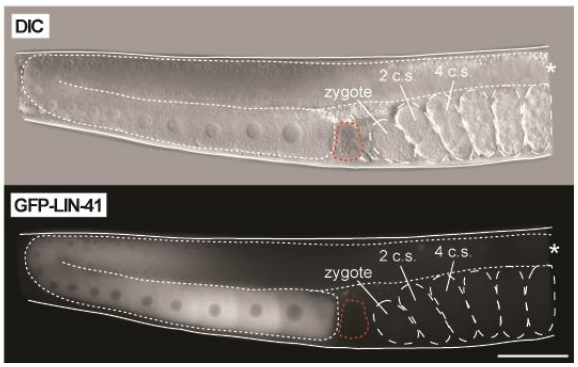

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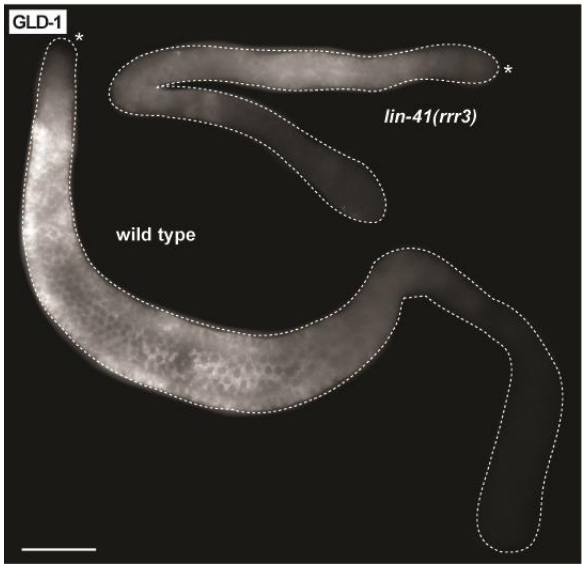

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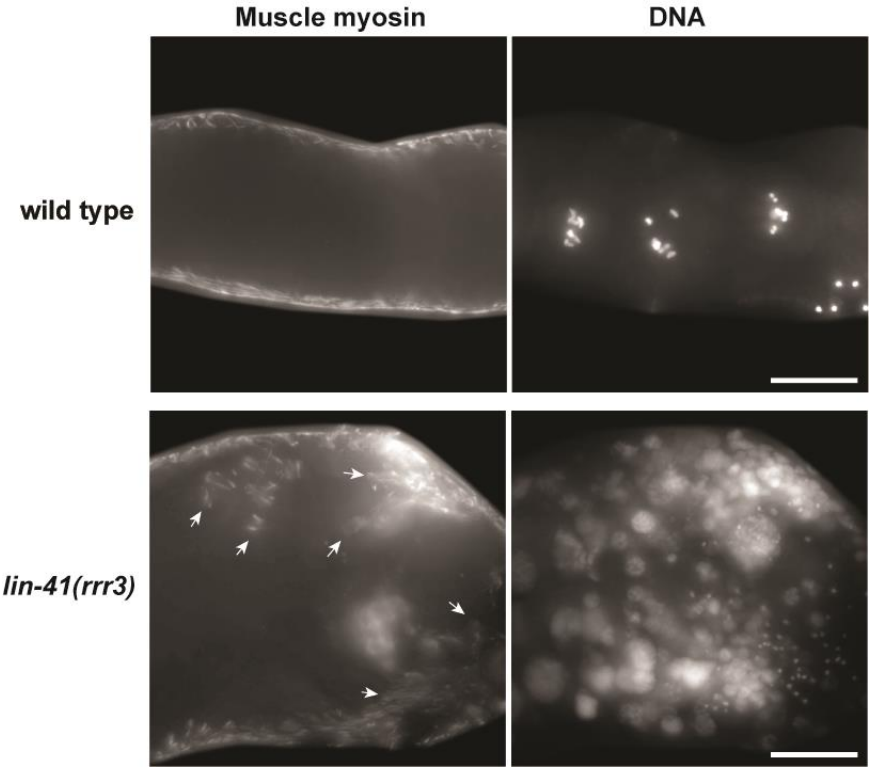

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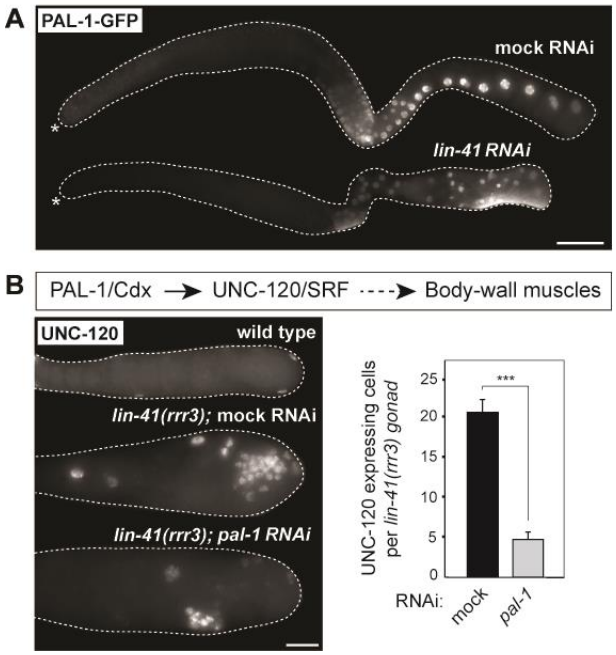

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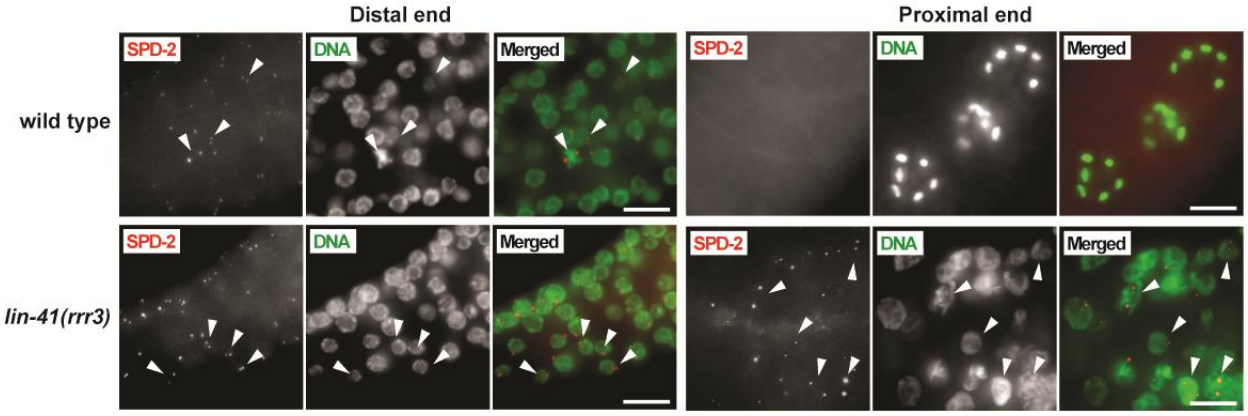

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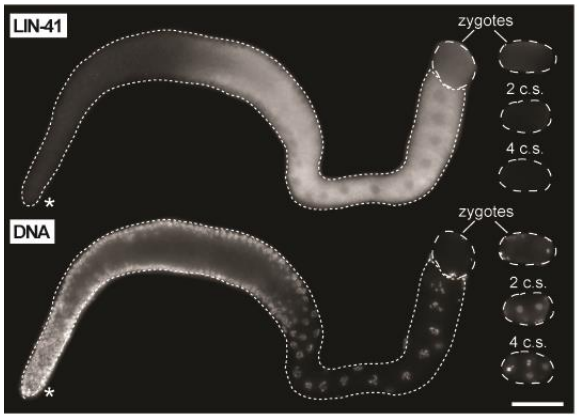

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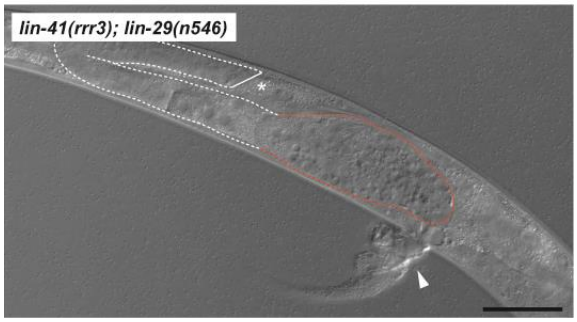

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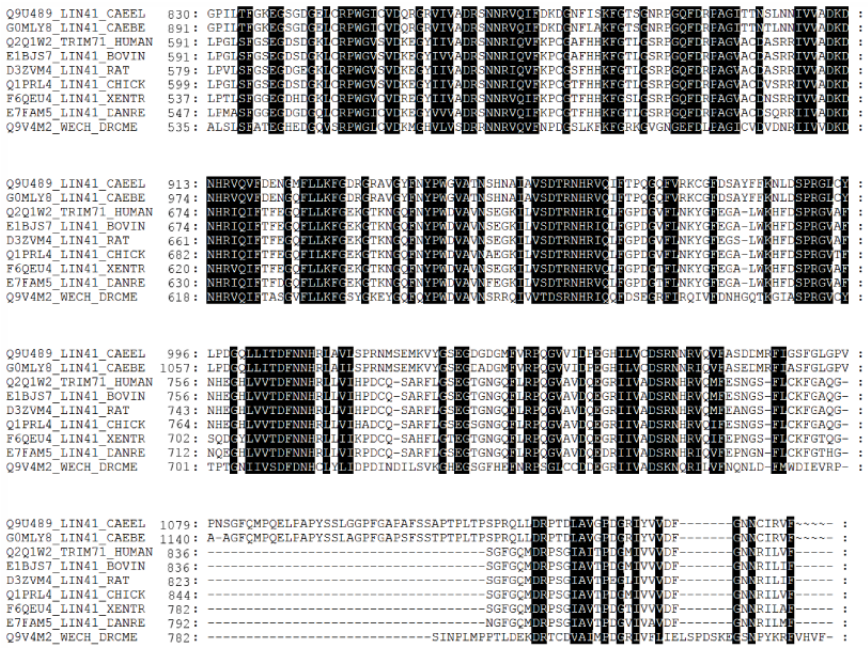
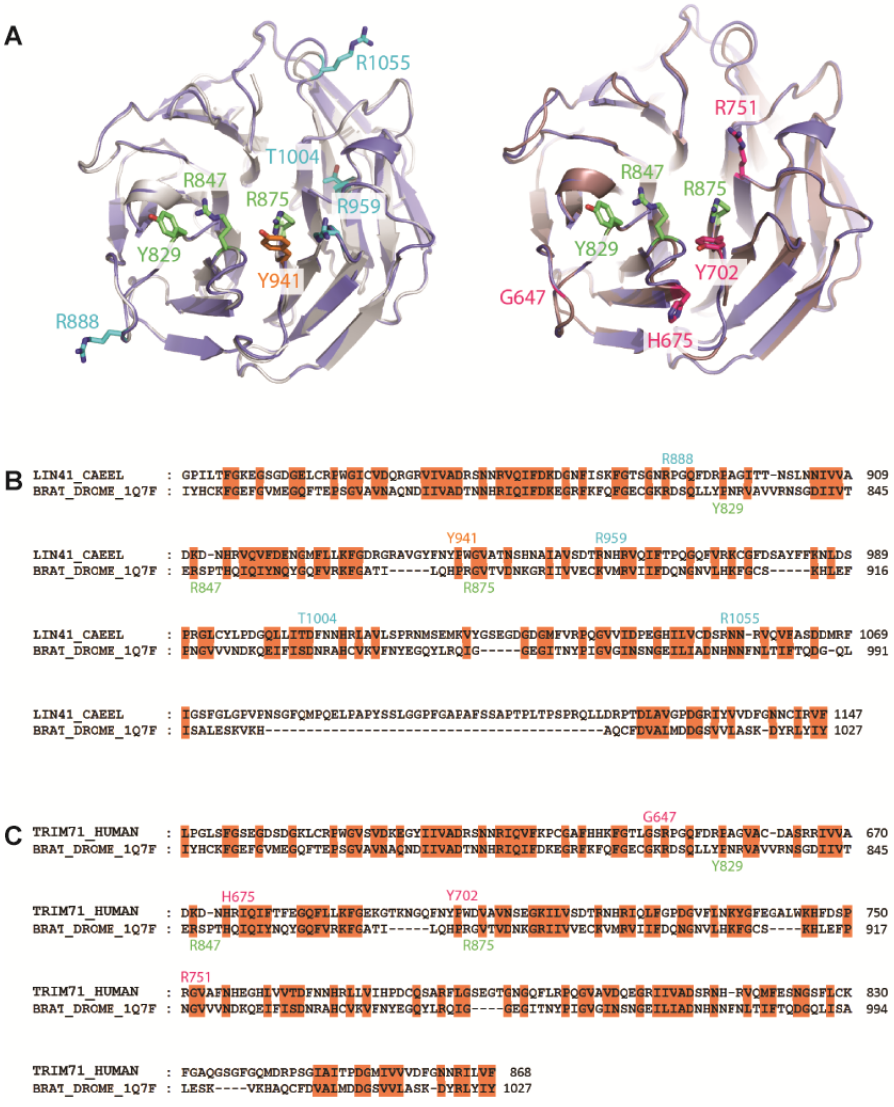


Tocchini_Fig S9



2. Recent insights into LIN-41 function in the germline

Cristina Tocchini, Andreas Arnold and Rafal Ciosk

In the paper Tocchini et al., 2014, we proposed the NHL repeats as the domain of LIN-41 which is directly involved in the control of pluripotency in the *C. elegans* germline and that it can do so independently of its ability to bind RNA. This hypothesis prompted us to identify through different approaches putative interacting partners of LIN-41 which could explain the teratoma phenotype. After several unsuccessful attempts to immunoprecipitate the FLAG- and GFP-tagged wild-type LIN-41 and identify which proteins could co-immunoprecipitate with it through mass spectrometry analyses, we decided to try an *in vitro* approach with a yeast-two-hybrid screen. LIN-41 full length was designed as the bait and it was screened against a yeast library designed on *C. elegans* prays. Unfortunately, the only "highly likely interactor" (class ID: A) identified in such screen resulted to be a vitellogenin (Fig. 2.1), which can be unlikely regarded as a candidate partner of LIN-41 in controlling pluripotency due to its lack of any catalytic or regulatory activities. Looking at the so-called "singletons" (class ID: C), we did find some candidates whose known functions might have had some sort of sense in the context of LIN-41 regulation of pluripotency in the germline. Specifically, these factors were: the chromatin remodeler EGL-27 (one of the components of the nucleosome remodeling and histone deacetylation (NURD) complex – Solari et al., 1999), the translational regulator EEF-2 (the homolog of the translation elongation factor 2 – Ofulue and Candido, 1991), the cell cycle controllers PLK-1 (a serine/threonine polo-like kinase – Chase et al., 2000) and PAA-1 (the structural subunit of protein phosphatase 2A (PP2A) – Janssens and Goris, 2001). Unfortunately, down-regulation via RNAi of the genes coding for such factors didn't show any remarkable change in the phenotypes of both wild-type and *lin-41(rrr3)* mutant carrying the EGA-GFP reporter on their back-grounds.

In parallel, we decided to further investigate the partial germline rescuing phenotype shown by the worm strain with our LIN-41^{Y941A} variant in the *lin-41(rrr3)* mutant back-ground (for simplicity, now on called "LIN-41^{Y941A} variant"), described in Tocchini et al., 2014. The fact that LIN-41, at least partially, co-localizes with P-granules (Fig. 2.2A), hinting for a role in RNA regulation in germ cells, gave us a further reason to analyze more in detail the LIN-41^{Y941A} variant and clarify what other phenotypes we could identify behind (and maybe directly causing) the more general partial rescue phenotype of the germline. For reasons which will become clear in chapter 2 of the appendix ("Mutants with EGA-positive oocytes"), we believe that the *in situ* hybridization is not a sensitive enough technique to detect small

amount of mRNA in germ cells. Therefore, to make sure that the EGA was really not occurring in the LIN-41^{Y941A} variant germlines (as shown in Fig. 7D of the attached manuscript, Tocchini at al., 2014), we decided to create a new EGA reporter, this time expressing mCherry-H2B and single-copy integrated on chromosome IV (all our *lin-41* transgenes are GFP-tagged and single-copy integrated on chromosome II). Interestingly, we could, indeed, detect EGA-mCherry-positive developing oocytes in all the analyzed LIN-41^{Y941A} variant animals (n = 33) (Fig. 2.2B). Another interesting observation in this regard has been made on the *lin-41(ma104)* mutant. The functionality of the filamin domain, suggested to be involved in post-transcriptional gene regulation in the human TRIM71 (Loedige et al., 2013), was supposed to be corrupted by de-fault in *lin-41(ma104)*, due to the 16 amminoacid insertion in the domain. We could not detect any ectopic EGA-GFP expression in these mutant gonads when grown at 20°C, but, apparently, the *lin-41(ma104)* behaves, at least to some extent, as a thermo-sensitive (TS) allele: when we tried to grow the worms at strict 25°C, all the gonads resulted to be EGA-GFP-positive and the brood size dramatically decreased to levels comparable to our LIN-41^{Y941A} variant (data not shown). Furthermore, both LIN-41^{Y941A} variant (100% penetrance, n = 18; Fig. 2.2C) and *lin-41(ma104)* (data not shown) could show a strong delay in (or even the absence of) centrosome elimination, indicated by the persistent presence of the centrosomal component SPD-2 in LIN-41^{Y941A} variant developing oocytes, when it would normally occur in the loop region in wild-type gonads. It is also important to note that some cells could show duplicated centrosomes, suggesting that, although they cannot complete it, they might try to enter mitosis. These data suggest that the putative role of LIN-41 in post-transcriptional gene regulation might be, indeed, important in *C. elegans* germlines, too, which was previously underestimated due to the use of a not sensitive enough technique to detect the EGA for the LIN-41^{Y941A} variant and to the fact that the *lin-41(ma104)* mutant was not supposed to behave as a TS.

To validate this hypothesis, we decided to generate more FLAG- and GFP-tagged single copy integrated domain mutants for LIN-41: mutants for BB1 and BB2, disrupting with point mutations the cysteine residues contacting zinc ions, as previously done with the RING domain; a deletion mutant for the CC; multiple point mutations affecting the NHL repeats to fully disrupt the binding to RNA, without altering the overall three-dimensional structure (same correspondent mutations used in Loedige et al., 2013 for the mammalian TRIM71). So far, we managed to create the LIN-41 mutant version of BB2 ("BB2*": C479S, C490S, C493S, C500S, C503S), a double- ("NHLx2": Y941A, R991A), a triple- ("NHLx3": G886D, Y941A, R991A) and a quadruple-point ("NHLx4": G886D, H914L, Y941A, R991A) mutants for the NHL domain (Fig. 2.3A). When crossed into the *lin-41(rrr3)* mutant back-ground we could not detect any abnormalities in the gonads of "BB2*", but all the three NHL mutants could show the germline

phenotype typical of null *lin-41* mutants (Fig. 2.3B). It is fair to stress out that the "NHLx2" does not show a proper localization in the gonad, but it seems to cluster in ectopic granules (data not shown), therefore the null phenotype could be due to the mis-localization of the protein and not to a specific loss-of-function in the domain. Although the other domain mutants are missing, the data provided by the new NHL mutants suggest that the LIN-41 post-transcriptional gene regulation is, indeed, essential in the germline and that the single point mutation in the LIN-41^{Y941A} variant was probably still able to, somehow, compensate the decrease in the affinity with RNA and show a partial loss-of-function. The case of the LIN-41^{Y941A} variant would not be the first one where a single point mutation in the RNA binding site is able to prevent a total loss-of-function phenotype: the hypomorfic allele of *gld-1*, *rrr1*, we identified in the original mutagenesis screen, does, indeed, prove that this hypothesis is most likely right.

Although direct evidences are missing, with these new data it seems that LIN-41 most likely acts as a post-transcriptional regulator. Given this assumption, we decided to look at the transcriptome profiles of wild-type versus *lin-41*-deficient animals. We did RNA-seq of whole young adult animals threated with mock or *lin-41 RNAi* and two technical replicates were made. To analyze the results, means of the two biological replicates were generated and the values were converted in $\log_2$ scale. The data were analyzed with scatter plots of the transcript abundances of mock versus *lin-41 RNAi* situation. Transcripts were considered up- (Fig. 2.4A) or down-regulated (Fig. 2.4B) in the *lin-41 RNAi* situation if their absolute values were respectively at least two-fold higher or lower of the mock ones. Such low differences were chosen based on the fact that the *lin-41 RNAi* was not very strong in that specific experiment. Considering that *lin-41 RNAi* also determines a somatic phenotype, we decided to look at how and how many germline transcripts (Scheckel et al., 2012) changed their expression levels (Fig. 2.4C). It came out that only 81 of the 2.416 up-regulated and 135 of the 731 down-regulated transcripts in the *lin-41 RNAi* situation were germline-enriched mRNAs (Fig. 2.4D). We, then, hypothesized that if LIN-41 more or less directly controls the expression levels of these transcripts, there might be, among the up-regulated ones, a repressor for the *lin-41* germline phenotype, similarly to what has been observed with *lin-29* in the soma (Slack et al., 2000). We selected roughly fifteen genes, based on the biological function, observed phenotype and putative interactors, and down-regulated them in a quick screen on the *lin-41(rrr3)*, seeking any kind of rescue of the germline mutant phenotype. Only one gene, *B0511.6*, could restore oocyte development to some extent (Fig. 2.4E). The fact that few animals presented restored oocytes (two out of eighteen) is probably due to the fact the knock-down of this gene determines additional phenotypes (larval arrest, absence of germlines and developmental delay).

As no TS mutant for such gene is available, we will need to test different RNAi condition to maximize the effect on the developmental stage we want to analyze. Anyway, *B0511.6* codes for a highly conserved DEAD-box helicase (Fig. 2.4F), which has been shown in yeast (named has1) to be involved in the biogenesis of ribosomal subunits (Emery et al., 2004) and in *D. melanogaster* (named Pitchoune) to be required for cell growth and proliferation (Zaffran et al., 1998), which perfectly fits with our observation.

ID	Prey-ID	uniprot ID	protein name (description)
A	pLexA-LIN41_prey43_fs-Gal-AD_E03	G8JY38	Protein VIT-2, isoform b OS=Caenorhabditis elegans GN=vit-2 PE=2 SV=1
A	pLexA-LIN41_prey76_fs-Gal-AD_A05	G8JY38	Protein VIT-2, isoform b OS=Caenorhabditis elegans GN=vit-2 PE=2 SV=1
A	pLexA-LIN41_prey80_fs-Gal-AD_B05	G8JY38	Protein VIT-2, isoform b OS=Caenorhabditis elegans GN=vit-2 PE=2 SV=1
C	pLexA-LIN41_prey02_fs-Gal-AD_C01	A8YPA8	Beta-actin (Fragment) OS=Chondrostoma olisiponensis GN=actb PE=3 SV=1
C	pLexA-LIN41_prey09_fs-Gal-AD_G01	G5EEW3	Protein F58A6.9 OS=Caenorhabditis elegans GN=msp-74 PE=4 SV=1
C	pLexA-LIN41_prey51_fs-Gal-AD_C04	J7RNK9	Protein EGL-27, isoform d OS=Caenorhabditis elegans GN=egl-27 PE=4 SV=1
C	pLexA-LIN41_prey_03_fs-Gal-AD_D01	O17038	Protein COL-143 OS=Caenorhabditis elegans GN=col-143 PE=4 SV=1
C	pLexA-LIN41_prey07_fs-Gal-AD_F01	O44144	Protein PERM-4 OS=Caenorhabditis elegans GN=perm-4 PE=4 SV=1
C	pLexA-LIN41_prey90_fs-Gal-AD_E05	O44871	Protein CLEC-150 OS=Caenorhabditis elegans GN=clec-150 PE=4 SV=2
C	pLexA-LIN41_prey10_fs-Gal-AD_H01	O76555	Protein MLT-10 OS=Caenorhabditis elegans GN=mlt-10 PE=4 SV=1
C	pLexA-LIN41_prey56_fs-Gal-AD_D04	P18947	Vitellogenin-4 OS=Caenorhabditis elegans GN=vit-4 PE=1 SV=3
C	pLexA-LIN41_prey47_fs-Gal-AD_G03	P29691	Elongation factor 2 OS=Caenorhabditis elegans GN=eef-2 PE=1 SV=4
C	pLexA-LIN41_prey28_fs-Gal-AD_B03	P34331	Isoform a of Serine/threonine-protein kinase plk-1 OS=Caenorhabditis elegans GN=plk-1
C	pLexA-LIN41_prey25_fs-Gal-AD_H02	P43509	Cathepsin B-like cysteine proteinase 5 OS=Caenorhabditis elegans GN=cpr-5 PE=2 SV=1
C	pLexA-LIN41_prey31_fs-Gal-AD_C03	P55956	Aspartic protease 3 OS=Caenorhabditis elegans GN=asp-3 PE=1 SV=2
C	pLexA-LIN41_prey15_fs-Gal-AD_C02	Q09456	Putative cuticle collagen 80 OS=Caenorhabditis elegans GN=col-80 PE=3 SV=1
C	pLexA-LIN41_prey12_fs-Gal-AD_B02	Q09543	Probable serine/threonine-protein phosphatase PP2A regulatory subunit OS=Caenorhabditis elegans GN=paa-1 PE=3 SV=2
C	pLexA-LIN41_prey24_fs-Gal-AD_G02	Q17411	Protein B0001.2 OS=Caenorhabditis elegans GN=B0001.2 PE=4 SV=2
C	pLexA-LIN41_prey20_fs-Gal-AD_E02	Q17802	Chondroitin proteoglycan 1 OS=Caenorhabditis elegans GN=cpg-1 PE=1 SV=1
C	pLexA-LIN41_prey04_fs-Gal-AD_E01	Q20287	Protein F41G3.6 OS=Caenorhabditis elegans GN=CELE_F41G3.6 PE=4 SV=2
C	pLexA-LIN41_prey23_fs-Gal-AD_F02	Q21966	Protein ASP-4 OS=Caenorhabditis elegans GN=asp-4 PE=3 SV=1
C	pLexA-LIN41_prey50_fs-Gal-AD_B04	Q8MXR8	Protein Y73B3A.18, isoform b OS=Caenorhabditis elegans GN=CELE_Y73B3A.18 PE=2 SV=1
C	pLexA-LIN41_prey89_fs-Gal-AD_D05	Q95ZM2	Protein F19F10.12 OS=Caenorhabditis elegans GN=CELE_F19F10.12 PE=4 SV=1
C	pLexA-LIN41_prey64_fs-Gal-AD_G04	Q9BHK7	Protein XRN-1 OS=Caenorhabditis elegans GN=xrn-1 PE=4 SV=3
C	pLexA-LIN41_bait01_Lexfow_A01	Q9U489	Protein lin-41 OS=Caenorhabditis elegans GN=lin-41 PE=2 SV=1
C	pLexA-LIN41_prey17_fs-Gal-AD_D02	Q9XWR7	Protein Y11D7A.3, isoform a OS=Caenorhabditis elegans GN=CELE_Y11D7A.3 PE=2 SV=1

Figure 2.1. List of candidate LIN-41 interactors based on a yeast-two-hybrid experiment: List of putative LIN-41 interactors from a yeast-two-hybrid screen from DualsystemsBiotech© using LIN-41 as a bait. The putative interactors are subdivided into three classes (A, B and C – first column) which are described in the materials and methods.

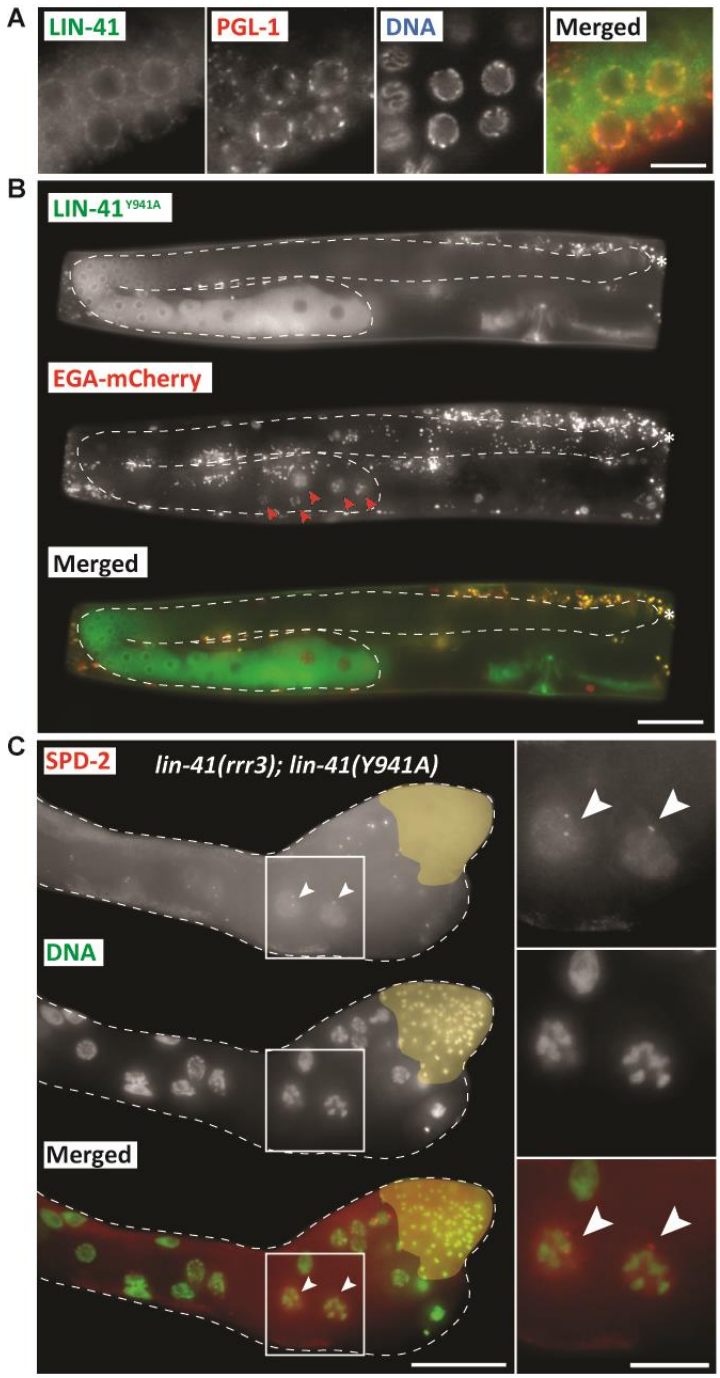


Figure 2.2. LIN-41 post-transcriptional gene regulation might have a role in the germline: **A.** Fluorescent micrographs of late pachytene nuclei of wild-type gonads immunostained for LIN-41 and the P-granule component PGL-1. The corresponding DAPI-stained nuclei and a merged image are also shown. Scale bar: 10 µm. **B.** Fluorescent micrographs of live *lin-41(rrr3)* mutant animals carrying the rescuing transgene coding for the GFP-tagged LIN-41^{Y941A} variant and the EGA-mCherry reporter. Red arrowheads point to EGA-mCherry-positive developing oocytes (100% penetrance, n = 33). Scale bar: 25 µm. **C.** Fluorescent micrographs of proximal gonads from *lin-41(rrr3)* mutant animals carrying the rescuing transgene coding for the LIN-41^{Y941A} variant and immunostained for the centrosomal component SPD-2. The corresponding DAPI-stained nuclei and merged image are also shown. Sperm is shaded in yellow. A zoom-in on a selected region (white-boxed) is shown on the right. Arrowheads point to proximal SPD-2-positive cells. All examined gonads show a delay in or absence of centrosome elimination in the proximal gonad (n =18). Scale bars: 25 (left) and 10 (right) µm.

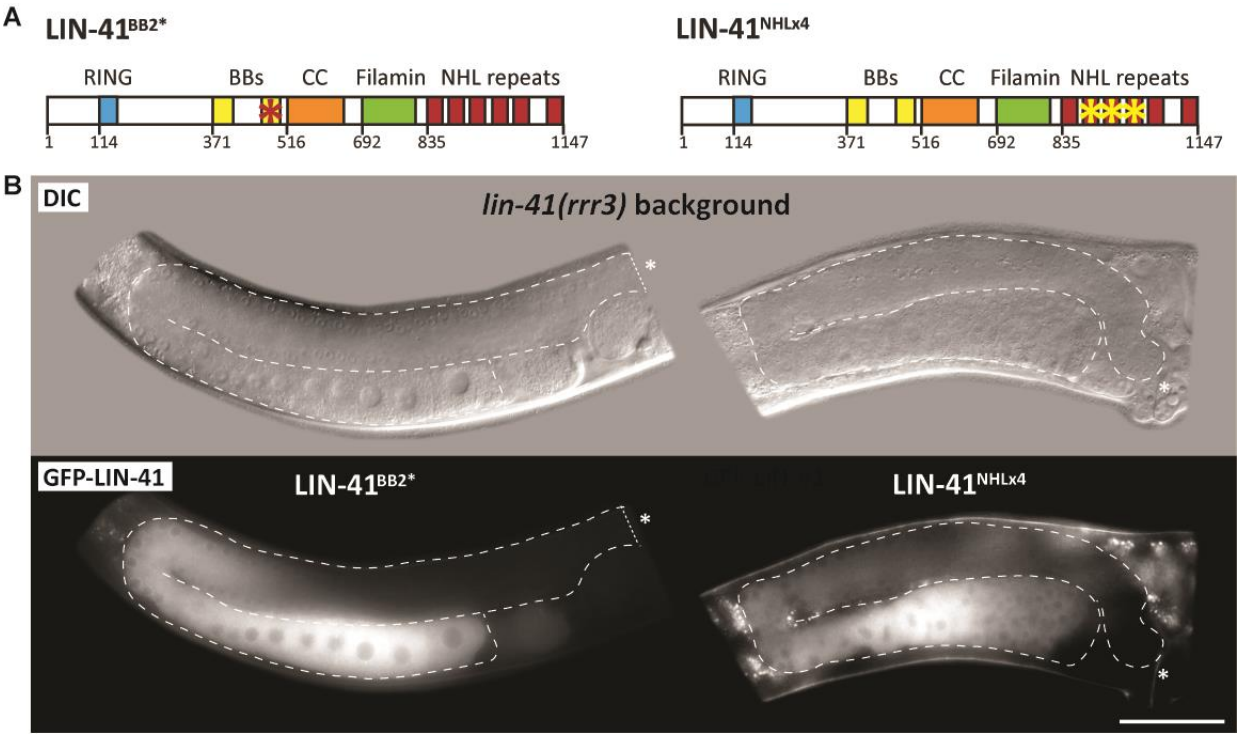


Figure 2.3. LIN-41 regulates pluripotency thank to the ability of its NHL repeats to bind RNAs: **A.** Schematic view of the LIN-41 domain structure with highlighted with asterisk the domains (or subdomains) affected by the point mutations. **B.** DIC (upper panel) and fluorescent (lower panel) micrographs of live *lin-41(rrr3)* mutant animals carrying GFP-tagged LIN-41 mutant variants (BB2* on the left and NHLx4 on the right) in their back-grounds. Scale bar: 25 μm.

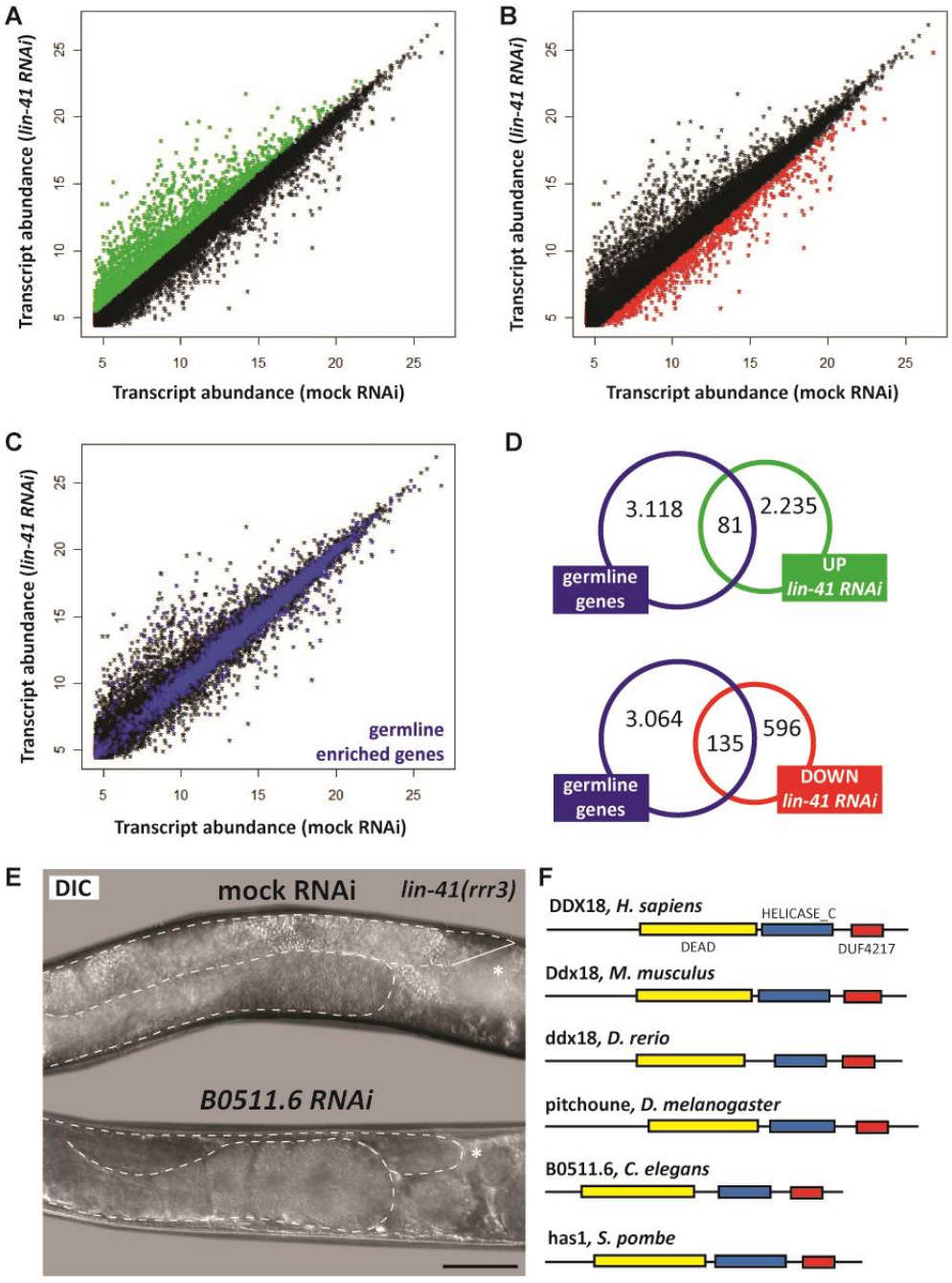


Figure 2.4. B0511.6 is a putative LIN-41 target which controls oogenesis: **A-C.** Scatter plots comparing whole animal transcript abundance in mock RNAi (x axis) versus *lin-41 RNAi* (y axis). In green (A), transcripts which are up-regulated more than two-folds in the *lin-41 RNAi* situation. In red (B), transcripts which are down-regulated more than two-folds in the *lin-41 RNAi* situation. In blue (C), germline-enriched genes. Values are in $\log_2$ scale. **D.** Identification of how many germline genes vary their expression levels in the *lin-41 RNAi* situation. **E.** DIC micrographs of live *lin-41(rrr3)* animals subjected to mock or *B0511.6 RNAi*. Two out of eighteen *B0511.6 RNAi* animals could show some extent of rescue of oocyte development. Scale bar: 25 µm. **F.** Conservation of B0511.6 protein from yeast to human.

Discussion

The recent evidences suggest that, as well as in the somatic cells, LIN-41 acts in the germline as an RBP. This function is essential in controlling the proper development of oocytes and in avoiding their precocious entrance into an embryonic state. The fact that our LIN-41^{Y941A} variant made us originally believe that LIN-41 function as an RBP was dispensable in controlling pluripotency was mainly due to a mis-interpretation of the partial loss-of-function phenotype this mutant showed. Single point mutants not always, indeed, determine a complete loss in the functionality of a certain domain, mostly when more than one amminoacid is involved in the interaction with either another factor or with nucleotide strands (*e.g.*, Daubner et al., 2014). The fact that the *lin-41(ma104)* could show a similar phenotype to our LIN-41^{Y941A} variant provided a further evidence that we might have been right with our hypothesis, until we realized that this mutant is behaving as a TS allele concerning the germline phenotype and that, therefore, it cannot be trusted as a mutant where the filamin domain function is fully abolished. With our new point mutants on the NHL repeats and their phenotypic readout, we have now the right tools to fully demonstrate the direct role of LIN-41 as an RBP and to identify which RNA targets it actually binds and potentially regulates.

If LIN-41 does, indeed, act as an RBP in oocyte development, post-transcriptional gene regulation appears, then, to be the sole mechanism which controls pluripotency in the germline, with GLD-1 and LIN-41 complementing each other's expression in the meiotic gonad (Fig. 2.5). When one of these two factors is missing, we assist to a precocious entrance of germ cells into a pluripotent state with their following differentiation and formation of a teratoma (Ciosk et al., 2006; Tocchini et al., 2014). GLD-1 has been shown to control the translation and stabilization (Scheckel et al., 2012) of around 1.000 mRNA targets (Wright et al., 2011) among whom there are transcripts coding for maternally provided embryonic cell fate determinants, cell cycle regulators and factors involved in oocyte development. The way GLD-1 is supposed to prevent the precocious entrance of germ cells into a pluripotent state was described in Biedermann et al., 2009, and re-proposed here (Fig. 2.5, middle panel). Based on that and on our newest preliminary data, we can propose a model for LIN-41 post-transcriptional regulation of pluripotency in developing oocytes. Oocytes have been shown to already contain factors whose mRNAs are GLD-1 targets (in grey or absent in Fig. 2.5, bottom panel): for example, maternally provided embryonic cell fate determinants, such as PAL-1 (Tocchini et al., 2014), cell cycle regulators which, in the *gld-1* mutant, prevent teratoma formation (CYE-1), as well as, of course, factors involved in oocyte development. Therefore, all the factors which, in the case of the *gld-1* mutant, are necessary for the entrance into a pluripotent state seem to be already present in developing oocytes. Given the different developmental context of germ cells, it can be that, in the case of the *lin-41* mutant, not CYE-1, but

another cell cycle regulator is responsible for the formation of the tumor. From our preliminary RNAi screen, it seems possible that this hypothetical cell cycle regulator is controlled, on its turn, by another RBP, B0511.6, whose expression, in wild-type oocytes, might be directly repressed by LIN-41 until the onset of embryogenesis. Therefore, in the *lin-41* mutant, the cell cycle regulation provided by B0511.6 would play a role as similar as CYE-1 in the *gld-1* mutant. The validation of B0511.6 as a LIN-41 target and the identification of the factor which directly controls the cell cycle in this context are definitely the priorities for the next future experiments.

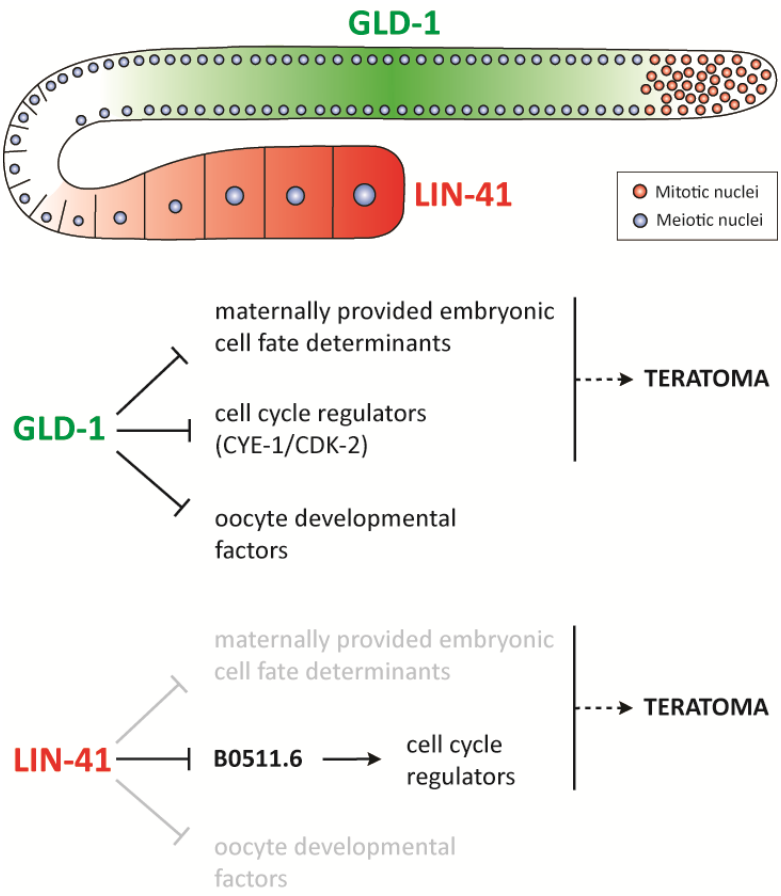


Figure 2.5. Proposed model through which GLD-1 and LIN-41 control germline development and pluripotency: Top: schematic view of the *C. elegans* gonad, showing mitotic and meiotic nuclei (red and blue dots, respectively) and expression patterns of the cytoplasmic proteins GLD-1 (green) and LIN-41 (red). Middle: modified model proposed by Biedermann et al., 2009, on how GLD-1 controls germline development and pluripotency. Bottom: model, we propose here, on how LIN-41 may control germline development and pluripotency, based on the GLD-1 model.

Appendix

1. Features of early embryonic genes

Cristina Tocchini, Dimosthenis Gaidatzis, Yanwu Guo and Rafal Ciosk

To get a better understanding on how the EGA is regulated, we used *C. elegans* as a model system to investigate if any common features among the early embryonic (E.E.) genes (*i.e.*, specifically transcribed from the 4-8 c.s.) exist and, if yes, how these similarities can be explained by a certain mechanism.

In order to identify which genes could be specifically considered as "early embryonic" to conduct our analyses on, we used the published available data on transcriptome profiles obtained from highly synchronized embryos during a time course experiment (Baugh et al., 2003). The original dataset provided absolute values in parts per million (ppm) for all the identified transcripts at the different time points, corresponding to a precise embryonic developmental stage. First of all, we had to discriminate which transcripts had to be considered maternally provided and which ones were not present up to the 4 c.s., when Pol II-dependent transcription just re-started. In order to identify a cut-off below which we could consider a gene as not expressed, we plot the data (in $\log_2$ scale) provided for the 4 c.s. in a histogram: the data were nicely showing a bimodal distribution where the minimum between the two picks corresponded to 3.75, which was used as a reference value to call a gene "not germline expressed" (first pick) or "germline expressed" (second pick) (Fig. 1.1A). After a first analysis, concerning how transcript abundance varied during the early steps of embryogenesis, we could distinguish three main types of transcripts: (i) never expressed, (ii) maternally provided and (iii) early embryonic. Transcripts classified as "never expressed" had a value in transcript abundance at the 4 c.s. below 3.75 (*i.e.*, "not germline expressed") and it remained constant in the following cell stages (*e.g.*, *daf-12*, blue line in Fig. 1.1B). Although we didn't further analyze this class of transcripts, we can predict that this group probably contains mRNAs which are expressed at specific time points and/or in specific cell types during development, but that, for sure, are not essential in the early steps of embryonic differentiation. The "maternally provided" transcripts have been previously described and divided into two classes, based on their expression pattern in the early embryo (Seydoux and Fire, 1994): the transcripts, belonging to class I, are those mRNAs which persist in all blastomeres and can be considered as housekeeping (*e.g.*, *tbb-2*, pink line in Fig. 1.1B), whereas mRNAs, which are degraded in the somatic blastomeres, but not in the P lineage, belong to class II (*e.g.*, *cye-2*, yellow line in Fig. 1.1B). For these mRNAs their transcript abundance at the 4 c.s. was more than 3.75 (*i.e.*, expressed) and it either remained constant (class I) or

decreased (class II) in the following cell stages. Finally, we called "early embryonic" those mRNAs whose transcript abundance at the 4 c.s. was less than 3.75 (*i.e.*, not expressed) but it was, then, increasing at least 2 folds (*i.e.*, a difference of 1 in $\log_2$ scale) from the 4 to the 8 c.s. (*e.g.*, *pes-10*, green line in Fig. 1.1B) and/or from the 8 to the 15 c.s. (*e.g.*, *tbx-38*, red line in Fig. 1.1B). The step from the 15 to the 26 c.s. was not taken into account as the data of the 26 c.s. did not significantly differ from the ones of the previous stage (data not shown), suggesting the occurrence of a transcriptional pause, probably caused by cellular rearrangements or by a transcriptional remodeling due to the onset of gastrulation. This analysis led us to identify a small group of 295 genes, representing the putative E.E. genes and we could confirm the reliability of the analysis by verifying the presence in this class of already known E.E. genes, such as *pes-10*, *vet-1* and *vet-6* (Seydoux and Fire, 1994).

Once we identified the early embryonic genes, we collected the informations regarding the following gene features from the website www.wormbase.org:

- Lengths of the following parameters: gene body, transcript, coding sequence, 5' and 3' UTRs, average exon;
- Number of exon/introns;
- Operon structure;
- Chromosome location.

Such informations were, then, analyzed comparing the E.E. genes against all genes (Fig. 1.2). This analysis could show some interesting features of *C. elegans* E.E. genes. First of all, their overall gene body length presented lower values than expected (Fig. 1.2A). As transcript (Fig. 1.2B), coding sequence (Fig. 1.2C) and UTR (Fig. 1.2D and E) lengths didn't show any significant change from the expected, the change in the gene body length must be due to the presence of fewer and/or shorter introns. It is interesting to note that the overall coding sequence length resulted unchanged (Fig. 1.2C), despite the higher average exon length (Fig. 1.2F): this fact can be easily explained by the lower amount of exons (Fig. 1.2G) in the E.E. genes, which further prove the presence of fewer introns. Furthermore, E.E. genes in operon structures resulted to be less than 3%, compared to the expected 15% (Fig. 1.2H). This difference results even more striking, considering that nearly 50% of the germline expressed genes, therefore those belonging to the direct previous developmental step, are belonging to operon structures (data not shown). Finally, looking at the chromosome preference of E.E. genes, we could see a striking bias towards chromosome II, on the expenses of mostly chromosomes IV and V (Fig. 1.2I). It is also interesting to notice that chromosome X gets immediately activated during early embryogenesis

after having been silenced in the germline. Therefore, from this analysis we could draw the following conclusions: (i) the introns of E.E. genes are few and probably relatively short; (ii) E.E. genes preferentially avoid operon structures; (iii) chromosome II is enriched in E.E. genes.

From this analysis we could draw different conclusions: first of all, we have been able to identify roughly 300 genes which specifically start to get transcribed at the EGA, corresponding to the 4-8 c.s. of the *C. elegans* embryo, *i.e*., when Pol II-dependent transcription re-starts after its inactivity during oogenesis. This first wave of transcription is followed by a period of unchanged transcriptome profile, corresponding to the time at which gastrulation occurs at the 26 c.s.. This "pausing" can be interpreted in two different ways, which are not mutually exclusive: the first hypothesis is that gastrulation events do not need a further acquirement of new transcripts and the early embryonic factors are necessary and sufficient for such event to occur. The fact that the embryo is able to reach its 100 c.s. without the synthesis of embryonic transcripts (*e.g*., through inhibition of AMA-1, the catalytic subunit of Pol II), but cannot undergo gastrulation (Edgar et al., 1994) would suggest that this hypothesis is probably right. On the other hand, similar modalities of transcriptional waves in early embryonic development have been shown for other organisms, too. People have speculated that such waves correspond to a first and minor wave of transcription, which is based on maternally provided factors, whereas a second and major wave occurs later on and it is based on the embryonic factors, newly synthetized by the embryo during the first wave (Tadros and Lipshitz, 2009). Such switch in transcriptional control might need one or two cell divisions to occur; therefore, what we observed at gastrulation would simply represent this period of change. Despite the fact that the group of E.E. genes does not mainly contain intron-less genes, the amount of exons, and therefore introns, is generally lower than expected. It has to be noticed that more than one third of these genes present either one or no intron. In addition to this consideration, the fact that a very low percentage of E.E. genes resides in an operon structure would further suggest that there might be some mechanism which promotes the transcription of genes presenting low complexity in their structure. This could also be an indirect effect of the cell cycle speed, as observed in *D. melanogaster* (De Renzis et al., 2007; Shermoen and O'Farrell, 1991), where longer and, in this case, more complex genes, which need to undergo several peri- or post-transcriptional modifications, get aborted. Unfortunately, no faster cell cycle has been reported to occur in the early *C. elegans* embryo, compared to later stages, making such a hypothesis inconsistent. That makes more probable, then, the possibility that maybe active mechanisms play a role in avoiding the expression of complex structured genes during EGA. Finally, we could show a chromosome bias for the E.E. genes, where more than double of the expected genes resides on chromosome II. Such a bias has never been

shown in other organisms, but it might represent a standardized way *C. elegans* evolutionary locates the genes belonging to a common class: a similar chromosome bias, in fact, has been shown to exist for germline expressed genes, which are enriched in chromosomes I and III (Gerstein at al., 2010). Although it is hard to hypothesize how and why these biases occur, one can speculate that it has something to do with chromatin localization inside the nucleus and/or gene duplication (many E.E. genes are coding for F-box proteins).

In addition to these features, we also analyzed the promoter sequences of the E.E. genes, seeking common motives which could infer putative binding sites for TFs specifically controlling the EGA, in a similar manner to what has been shown for the TAGteam and Zelda in the early embryonic genes of *D. melanogaster* (De Renzis et al., 2007; ten Bosch et al., 2006). The analysis was conducted on all the promoters (defined from 2.000 bp upstream and 200 bp downstream the ATG) of the genes we identified as E.E. using the software Hypergeometric Optimization of Motif EnRichment (HOMER, Heinz et al., 2010) (Fig. 1.3). Strikingly, the top hit can be identified in nearly 50% of the analyzed promoters and such motif occurs at least once in all the promoters we commonly use to address the EGA (*vet-1*, *vet-4*, *vet-6* and *pes-10*), providing a good candidate DNA binding site for the identification of a putative EGA-specific TF. As mentioned in the introduction, different levels of regulation can control the EGA. With this E.E. promoter analysis, we suggest an additional level of regulation to what it has been previously shown by Guven-Ozkan et al., 2008. During oogenesis, the localization of the basal transcription machinery right on the promoters of the E.E. genes might be coordinated by a TF which binds to one the sequences we identified in the analysis. At the 4 c.s., then, TAF-4 will reach the transcription machinery and start the EGA (Guven-Ozkan et al., 2008). Further analyses will make us able to identify the first example of such a TF in *C. elegans* and to ask for its conservation in higher eukaryotes.

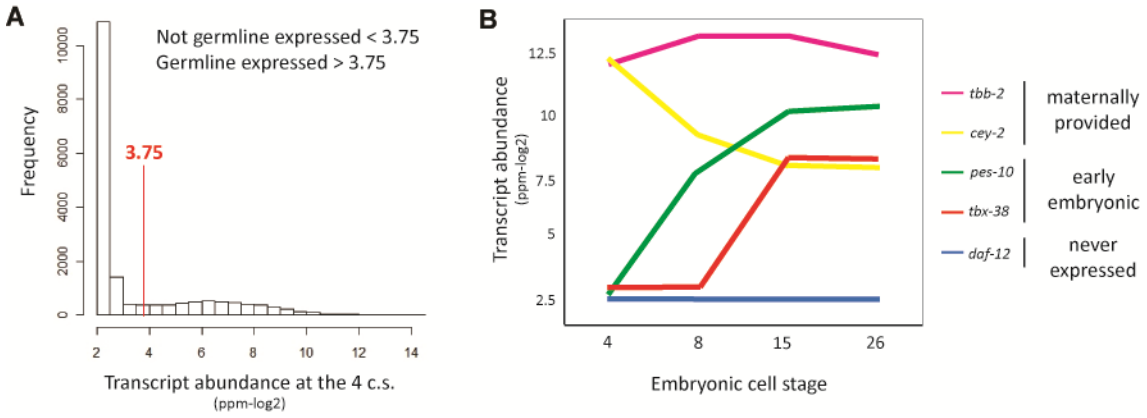


Figure 1.1. Identification of the early embryonic genes: **A.** Graph representing the bimodal distribution of transcript at the 4 c.s.. On the x axis: the values in parts per million (in $\log_2$ scale) of transcript abundance; on the y axis: the frequencies of the transcript occurrence. In red: the minimum of the distribution (3.75). Transcripts below this value are considered "not germline expressed", above "germline expressed". **B.** Graph representing transcript abundance changes (y axis) over embryonic cell stages (x axis). Examples for each kind of transcript reported in the text are shown.

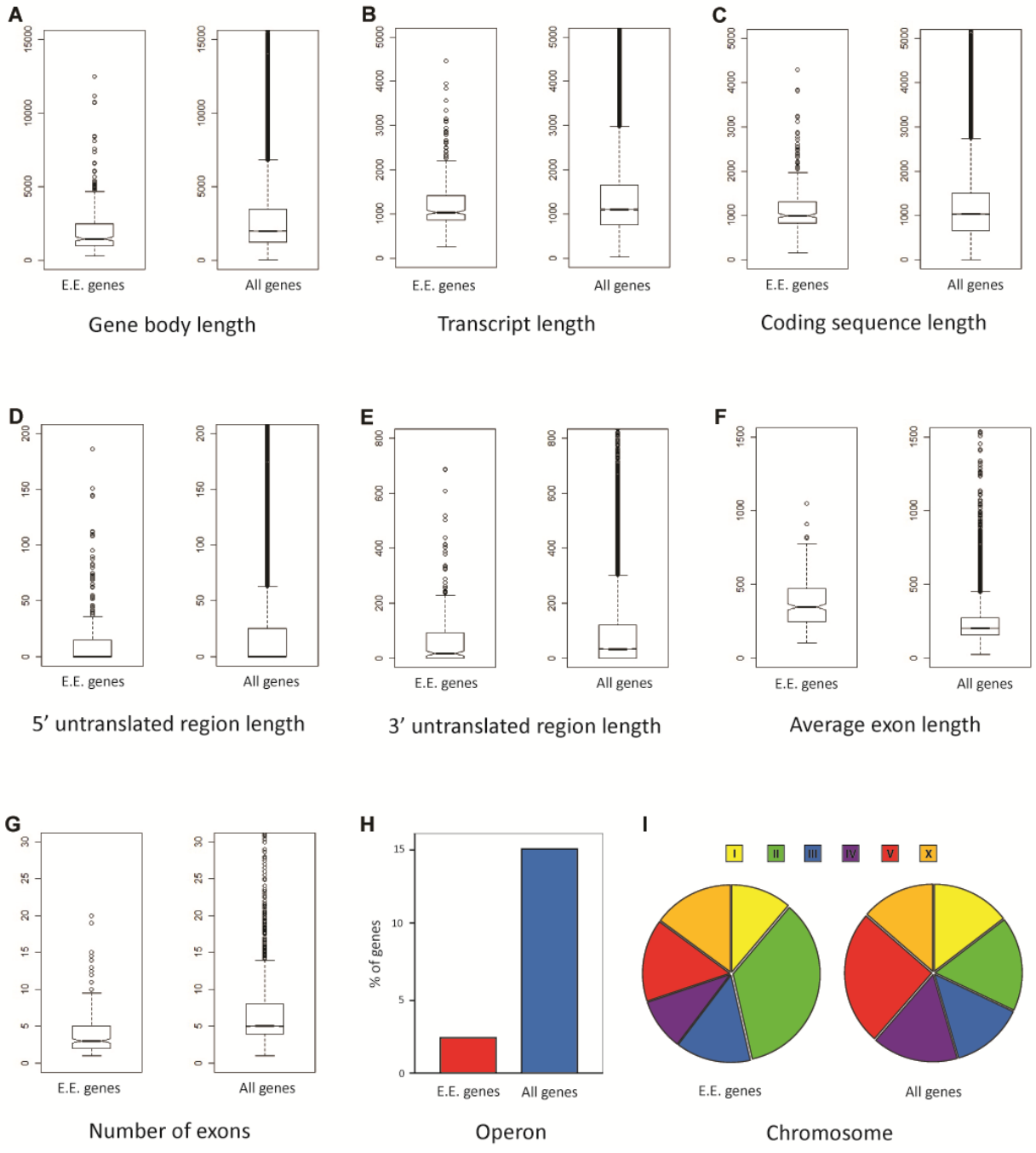


Figure 1.2. Comparison between E.E. genes and the rest (all genes) for different genetic features: **A-G.** Box plots comparing the corresponding genetic feature. **H.** Column chart showing the percentage of genes belonging to an operon structure. **I.** Pie chart showing the percentage of genes belonging to each chromosome.

Rank	Motif	P-value	log P-pvalue	% of Targets	% of Background
1		1e-34	-7.926e+01	47.95%	16.55%
2		1e-25	-5.885e+01	14.04%	1.51%
3		1e-23	-5.448e+01	20.21%	4.00%
4		1e-20	-4.713e+01	14.73%	2.34%
5		1e-19	-4.509e+01	6.51%	0.25%
6		1e-17	-4.026e+01	5.48%	0.18%
7		1e-16	-3.885e+01	7.88%	0.64%
8		1e-14	-3.430e+01	20.55%	6.38%
9		1e-14	-3.325e+01	6.16%	0.43%
10		1e-12	-2.975e+01	5.82%	0.45%
11		1e-12	-2.925e+01	5.48%	0.39%
12		1e-12	-2.920e+01	6.16%	0.55%
13		1e-12	-2.864e+01	22.95%	8.78%
14		1e-12	-2.796e+01	4.11%	0.17%
15		1e-12	-2.784e+01	9.59%	1.74%

Figure 1.3. Identification of candidate consensus sequences as putative binding sites for E.E.-specific TFs: List of the top fifteen hits from a query searching for common motives among the promoters of the 295 E.E. genes using the HOMER software.

2. Mutants with EGA-positive oocytes

Cristina Tocchini, Susanne Finger, Dimosthenis Gaidatzis and Rafal Ciosk

As previously mentioned in the introduction section, an EMS-based mutagenesis screen was conducted on a *C. elegans* strain carrying an EGA-GFP reporter seeking mutant animals which could precociously express GFP in germ cells instead of embryos. In this chapter data concerning the mutants belonging to what we called "class I" mutants will be described and discussed.

Two mutant strains, respectively called *rrr1* and *rrr2*, identified in the screen, could show similar phenotypic features, which prompted us to make them belong to the same class. Through complementation group analyses, we could identify *rrr1* as a *gld-1* allele and sequencing data could tell us that this was a new point mutation in the coding sequence affecting the proline residue in position 228 (P228S) of the GLD-1 protein (Galarneau and Richard, 2009; Ryder et al., 2004). More specifically, this residue is located the GXXG linker in between α-helix 1 and 2 of the KH domain and it directly recognizes and binds the uridine of the GLD-1 binding motif on the target mRNAs (Galarneau and Richard, 2009; Ryder et al., 2004; Wright et al., 2011). The consequences determined by this hypomorfic GLD-1 allele on the post-transcriptional regulation of its targets have been characterized and described in Daubner et al., 2014 and they won't be further discussed here. Concerning the *rrr2* mutant, it has been identified as a new allele for the gene *drh-3* through whole genome sequencing (WGS). Complementation group analyses and direct sequencing of the *drh-3* locus could confirm the WGS data which identified a point mutation in the acceptor splicing site of intron 11 (Fassnacht et al., in preparation). The detailed characterization of the *drh-3* gene and the role of the pathway it belongs to, concerning the regulation of the EGA, will be extensively described in Fassnacht et al., in preparation, and, therefore, only a subset of the results concerning such gene will be taken into account in this section.

Although *gld-1(rrr1)* and *drh-3(rrr2)* could show an overall normal gonad, minor defects in EGA-GFP-positive oocytes could be identified which, presumably, determined the sterility of the animals (Fig. 2.1A). Accordingly to the precocious EGA-GFP expression in mutant oocytes, the endogenous gene, whose regulatory sequences were used to create the EGA-GFP reporter, *vet-4*, was also found to be expressed in mutant gonads via RT-qPCR (Fig. 2.1B). In addition to *vet-4*, we could verify the expression of other early embryonic genes and also detect Hox transcripts which, in normal development, start to be expressed during gastrulation (26 c.s.) (Fig. 2.1B). Differently from the class II mutants, the class I

mutants showed no or not significant expression of somatic lineage-specific genes (Fig. 2.1C) and no terminally differentiated cells could be detected in their germlines (data not shown). These data could show that class I mutant germ cells are able to undergo the EGA and transcribe genes expressed during the very first steps of embryonic cell commitment, but are not able to terminally differentiate into any somatic cell.

With the will to understand why class I mutant germ cells are not able to terminally differentiate into somatic cells, despite their entrance into an embryonic state, we checked the state of Pol II-dependent transcription in mutant oocytes, testing the transcription-initiating phosphorylation of serine 5 (Ser5P) within the C-terminal domain (CTD) of Pol II (Seydoux and Dunn, 1997). As mentioned in the introduction, wild-type oocytes undergo repression in Pol II-dependent transcription, feature which is not observed in the class II mutant proximal gonads. Despite the fact they are EGA-GFP-positive, the class I mutant oocytes showed a complete (*drh-3(rrr2)*, data not shown) or a major (*gld-1(rrr1)*, Fig. 2.2A) inhibition of Pol II-dependent transcription, overall comparable to the wild-type situation. The presence of non-germline transcripts, detected via RT-qPCR, in the mutant gonads and the expression of EGA-GFP in their oocytes made us wandering how these two observations could be compatible with the absence of Pol II-transcription in their proximal gonads. By *in situ* hybridization, we could not, indeed, detect the *vet-4* transcript in the oocytes of *drh-3(rrr2)*, but only in the loop region, where Pol II-dependent transcription is still active. These data suggest that despite the presence of EGA-GFP in the mutant oocytes, the EGA itself, *i.e*., the actual transcription of early embryonic genes, occurs only before the oocytes develop. It is important to note that only 3 out of 20 *drh-3(rrr2)* and none of the *gld-1(rrr1)* gonads gave us a positive signal for the presence of the *vet-4* transcript. Given the very low amount of EGA-GFP observed and considering the small region where, presumably, the embryonic genes are transcribed, we fear that an *in situ* hybridization is not sensitive enough in this context to detect the aberrant transcripts which can, instead, be monitored with a more sensitive technique, such as RT-qPCR (Fig. 2.1B). For further analyses and to confirm this hypothesis, the use of a more sensitive technique, such as single molecule fluorescence *in situ* hybridization, is recommended.

As GLD-1 and DRH-3 belong to different pathways, we thought we could still use the similarities in their mutant phenotypes to understand which factors specifically regulate the EGA. We decided to analyze the germline transcriptome profiles of the two class I mutants with a microarray experiment. Transcripts were extracted from fifty gonads for each sample (wild-type, *gld-1(rrr1)* and *drh-3(rrr2)*) and two biological replicates were made. To analyze the results, means of the two biological replicates were

generated and the values were converted into $\log_2$ scale. The data were analyzed with scatter plots of the transcript abundances of wild-type versus mutant situations. Transcripts were considered up- or down-regulated in the mutant situation if their absolute values were respectively at least three-fold higher or lower than the wild-type ones (Fig. 2.3A). From the lists of up- and down-regulated transcripts in the two mutant situations, we extrapolated those mRNA which are up- (51 transcripts) and down-regulated (87 transcripts) in both mutants, trying to identify the factor(s) responsible for the onset of the EGA (Fig. 2.3B). Up-regulated transcripts were regarded as candidate activators for the EGA, whereas down-regulated ones as candidate suppressors. To test them we, therefore, conducted RNAi experiments for the up-regulated transcripts on the two mutant strains, seeking mutant gonads which could not express the EGA-GFP anymore. For the down-regulated transcripts, instead, RNAi experiments were done on the wild-type EGA-GFP reporter strain, having, in this case, ectopic EGA-GFP expression in the gonads as a readout. Unfortunately, in both experiments single gene knock-down didn't lead to any of the hypothesized phenotypes, suggesting that either more than one gene or pathway is involved in the regulation of the EGA or our approach was based on wrong assumptions and maybe the EGA is mis-controlled in different ways in the two mutant situations.

The mutants belonging to the class I provide an interesting resource to study how the EGA is controlled: in fact, differently from the class II mutants and normal embryogenesis, in their gonads we assist to the onset of the EGA, without any further cell differentiation. Despite the fact that the microarray experiment didn't allow us to identify any common players which can explain the occurrence of the EGA in both mutant gonads, it does not mean that there are none, but simply that probably we didn't use the right technique to address the problem. The microarray demonstrated from the beginning to be a not sensitive enough technique to detect small changes on transcript abundance for genes which are not normally expressed in the wild-type gonad. For example, despite the fact that we could validate the presence of early embryonic and Hox transcripts via RT-qPCR in the mutant samples used for the microarray (data not shown), *vet-1* was the only mRNA which could be shown to be up-regulated with the microarray data (Fig. 2.3B). Therefore, other experiments, such as RNA-sequencing (RNA-seq – more sensitive than a microarray) and ribosome profiling, maybe in a time-course, would provide further and more precise informations which may help in dissecting how embryonic gene transcription is maintained repressed in the germline. On the other hand, at the state of the art, we also cannot completely rule out the possibility that multiple pathways play a role in controlling the EGA in germ cells and that we are affecting two different ones in the two mutants, without having a change in the readout.

A possible explanation why the mutant EGA-GFP-positive oocytes cannot undergo somatic differentiation is that as soon as oocytes start developing, Pol II-dependent transcription gets inhibited and, therefore, abolishes embryonic mRNAs to get transcribed. To test this hypothesis, one would need to create an inducible and catalytically active Pol II (mutations: S2E and S5E of the CTD) in mutant oocytes (germline-specific promoter: *mex-5*) and see what happens to the oocyte fate once it is induced. On the other hand, we also need to take into account that there are other events which occur during the OET which allow the embryo to continue its development: one for all is the oocyte maturation which, on its turn, provides the signal for maternal factor degradation, which could be a limiting factor for the final somatic differentiation in the context of the class I mutant oocytes. It is also true that if we do the opposite experiment of what has just been proposed and we inhibit Pol II-dependent transcription in class II mutant teratomous germlines, through RNAi of the gene which code for the catalytic subunit of Pol II (*ama-1*), we do actually see at least a partial inhibition of somatic differentiation and promotion of oogenesis (Fig. 2.4). These data suggest that, indeed, transcriptional inhibition is, at least in part, important in promoting oocyte development and to avoid somatic differentiation, as already demonstrated for the P lineage during embryogenesis (Seydoux et al., 1996).

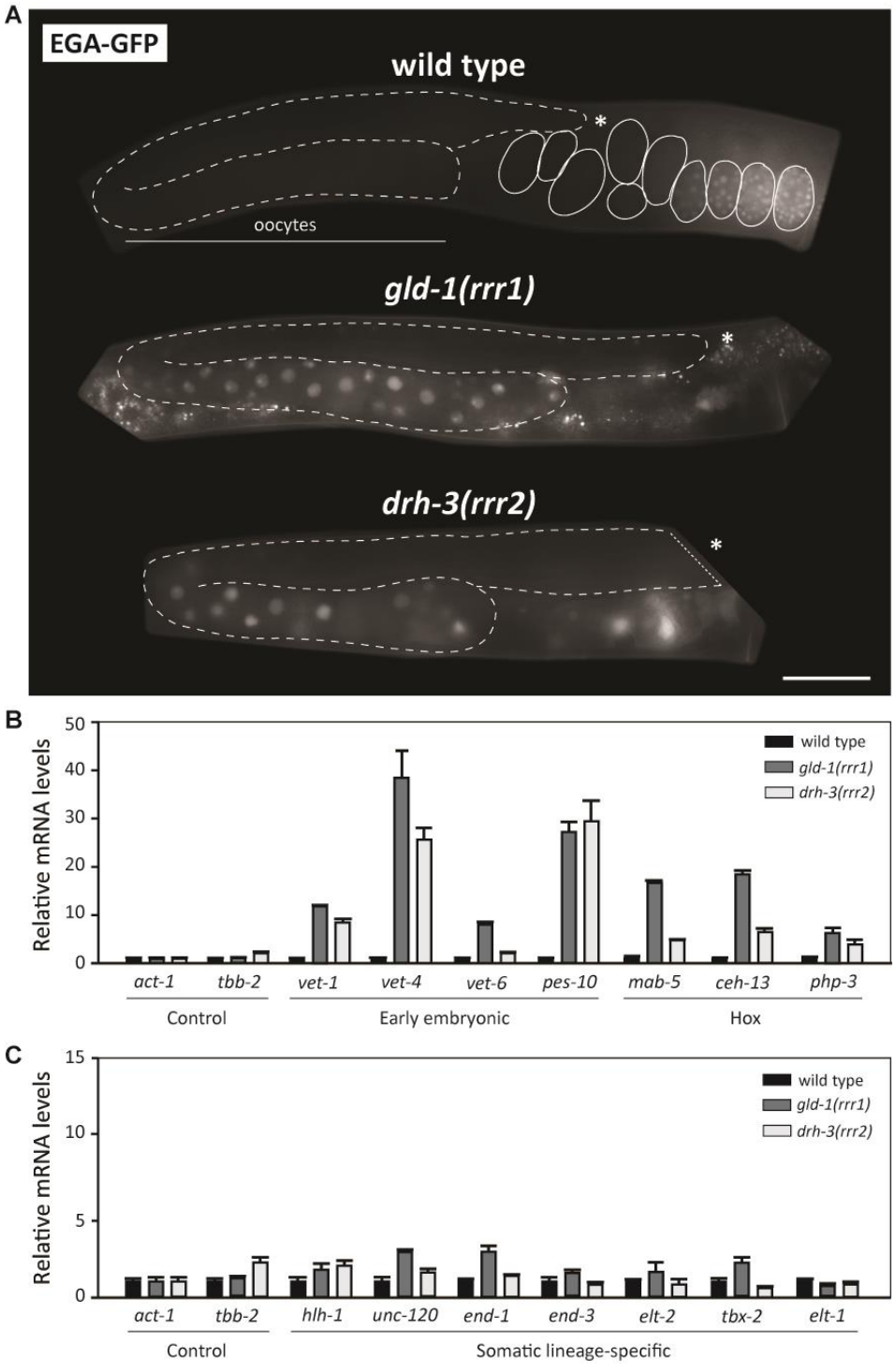


Figure 2.1. Class I mutant phenotypes: **A.** Fluorescent micrographs of live animals expressing EGA-GFP. Here as well as in all the other figures, gonads are outlined with dashed lines and embryos (wild-type) with continuous lines. Asterisks mark the distal end of the gonad and oocytes in the wild-type are indicated. Wild-type embryos show EGA-GFP from the 16 c.s.. *gld-1(rrr1)* and *drh-3(rrr2*) mutants abnormally express EGA-GFP in their oocytes. Such phenotypes had a 100% penetrance (n > 100 for both mutants). Scale bar: 25 µm. **B-C.** Detection of additional EGA, Hox and somatic lineage-specific transcripts in mutant gonads by RT-qPCR. "Early embryonic" are mRNAs normally expressed in the embryo following the EGA (4-8 c.s.), whereas "Hox" expression follows gastrulation (26 c.s.) (B). "Somatic lineage-specific" indicates mRNAs expressed in somatic cell lineages: muscle (*hlh-1* and *unc-120*) or gut/pharynx (*end-1*, *end-2*, *elt-2*, *tbx-2* and *elt-1*) (C). Each bar represents the mean of three independent biological replicates and the error bars represent the standard error of the mean (SEM).

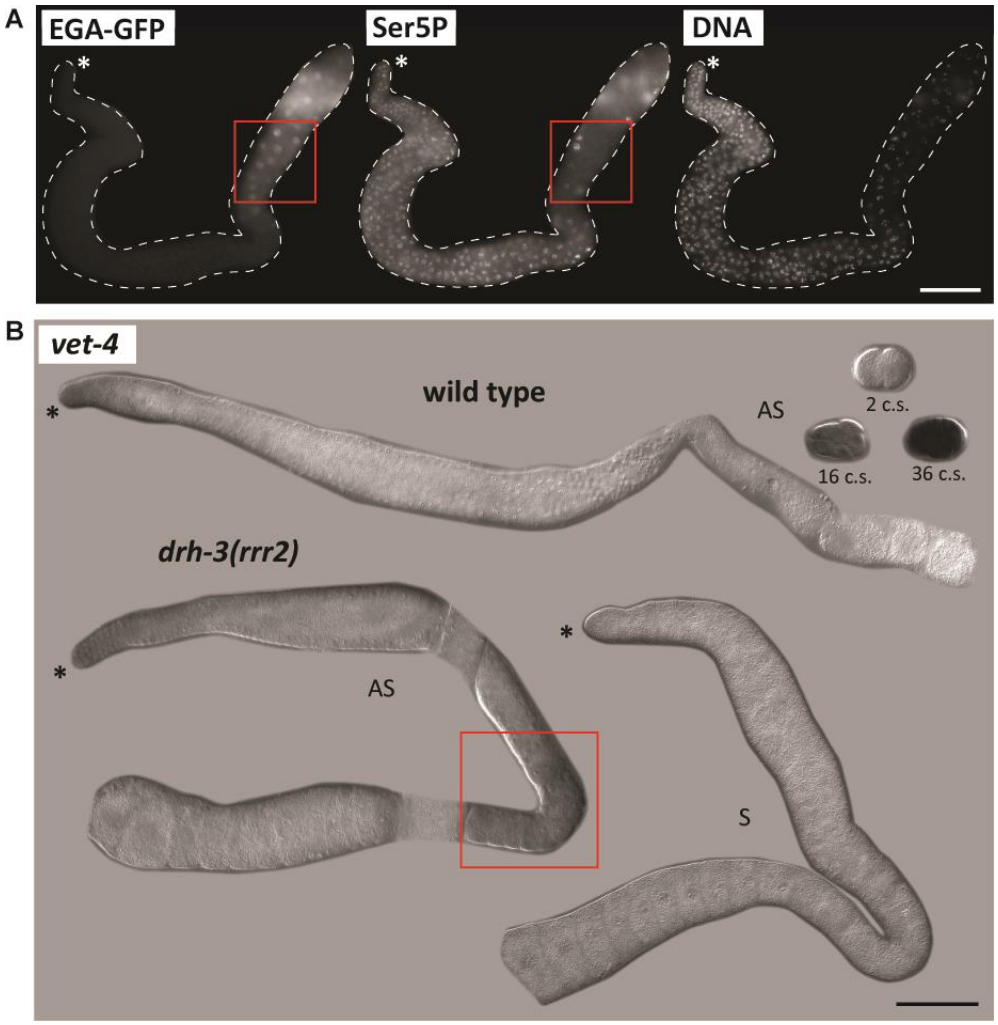
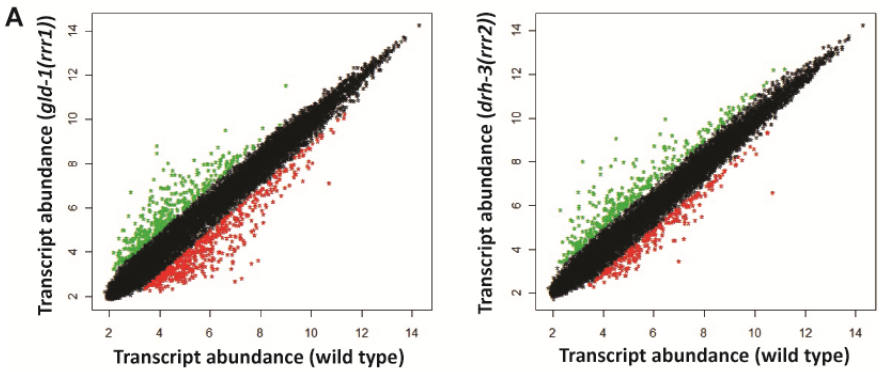
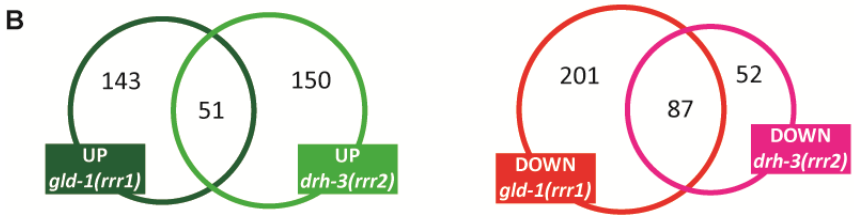


Figure 2.2. Pol II-dependent transcription is inhibited in mutant oocytes: **A.** Fluorescent micrographs of *gld-1(rrr1)* gonads immunostained for the transcription-activating phosphorylation of Ser5 within the Pol II CTD (Ser5P). This phosphorylation is known to be absent in wild-type oocytes and results absent from most of the EGA-GFP-positive mutant oocytes of *gld-1(rrr1)* – red boxed (n = 26). The corresponding DAPI staining is shown on the right. Scale bar: 25 µm. **B.** *In situ* hybridization against the endogenous *vet-4* transcript. Light micrographs of wild-type and *drh-3(rrr2)* gonads and wild-type embryos (at the indicated cell stage), which were hybridized with antisense (AS) probes for the *vet-4* mRNA. *drh-3* gonads were additionally hybridized with sense (S) probes. In the wild-type situation *vet-4* mRNA can be detected in embryos after the 4 c.s.; in *drh-3(rrr2)* mutant gonads a faint signal can be detected in 3 out of 20 gonads specifically in the loop zone (red box). Scale bar: 25 µm.





Common up-regulated transcripts

B0303.14	*EEED8.12*	*mif-1*	*T16G12.4*	*Y39G10AL.1*
B0511.11	*F08G2.4*	*mrp-8*	*T22B7.7*	*Y41C4A.13*
C13C12.2	*F28F8.7*	*npr-17*	*T24C4.2*	*Y45F3A.4*
C27D8.2	*F28H1.1*	*pmk-1*	*tag-276*	*Y51H4A.4*
C34F6.6	*F54C4.4*	*scrm-4*	*ttr-45*	*Y54F10BM.13*
cdr-4	*F58E10.7*	*set-12*	*unc-27*	*Y54G9A.4*
ceh-20	*hlh-3*	*skr-7*	*vet-1*	*Y65A5A.1*
ceh-91	*K03A11.1*	*spe-15*	*VT23B5.1*	*Y69A2AR.31*
cex-1	*K10G9.2*	*T05G5.1*	*W06D11.2*	*Y71F9B.8*
cpn-4	*kin-34*	*T13C2.7*	*Y116A8C.22*	*Y71G12B.30*
dhs-21				

Common down-regulated transcripts

alg-1	*dpy-19*	*gst-44*	*pzf-1*	*T19C3.4*
aps-1	*dre-1*	*H03A11.1*	*R04B3.2*	*T24E12.10*
arrd-18	*erd-2*	*H04M03.3*	*R04D3.4*	*T28C6.3*
bed-3	*F02H6.2*	*haf-7*	*R05H11.1*	*tag-123*
C06A6.5	*F08B1.3*	*K01G12.3*	*R08B4.3*	*unc-14*
C06H2.7	*F14H12.6*	*K04C1.5*	*rhy-1*	*W06D11.3*
C13G3.1	*F22E5.17*	*K07E3.1*	*rpm-1*	*wts-1*
C16D9.6	*F31D4.8*	*K09E3.6*	*sago-1*	*Y37F4.6*
C17H11.2	*F31E8.4*	*K10B4.3*	*set-19*	*Y38F1A.8*
C50E3.11	*F40G12.11*	*K12B6.8*	*sid-1*	*Y44A6C.1*
C50E3.13	*F47G3.3*	*lrp-2*	*sor-3*	*Y4C6A.3*
cdc-25.2	*F47G4.2*	*mboa-7*	*T01D1.4*	*Y73B6BL.30*
ced-8	*F49E12.6*	*nhr-138*	*T02C1.2*	*Y75B8A.6*
D1005.1	*F54F7.2*	*nhr-161*	*T04G9.1*	*ZK1098.7*
D1014.2	*F59A3.1*	*nhr-17*	*T09B9.1*	*ztf-22*
D1022.4	*frm-7*	*nhr-70*	*T14B4.8*	*ztf-25*
ddo-3	*gly-13*	*pyr-1*	*T14F9.2*	*ztf-6*
dhs-19	*gst-16*			

Figure 2.3. Microarray analysis of the germline transcriptomes of the class I mutants: **A.** Scatter plots comparing germline transcript abundance in wild-type (x axis) versus *gld-1(rrr1)* (y axis, left panel) or *drh-3(rrr2)* (y axis, right panel). In green, transcripts which are up-regulated more than three-folds in the mutant situation; in red, transcripts which are down-regulated more than three-folds in the mutant situation. Values are in $\log_2$ scale. **B.** Identification of common up- and down-regulated transcripts in both mutants. Lists of the 51 commonly up- (green boxed) and the 87 commonly down-regulated (red boxed) transcripts is provided.

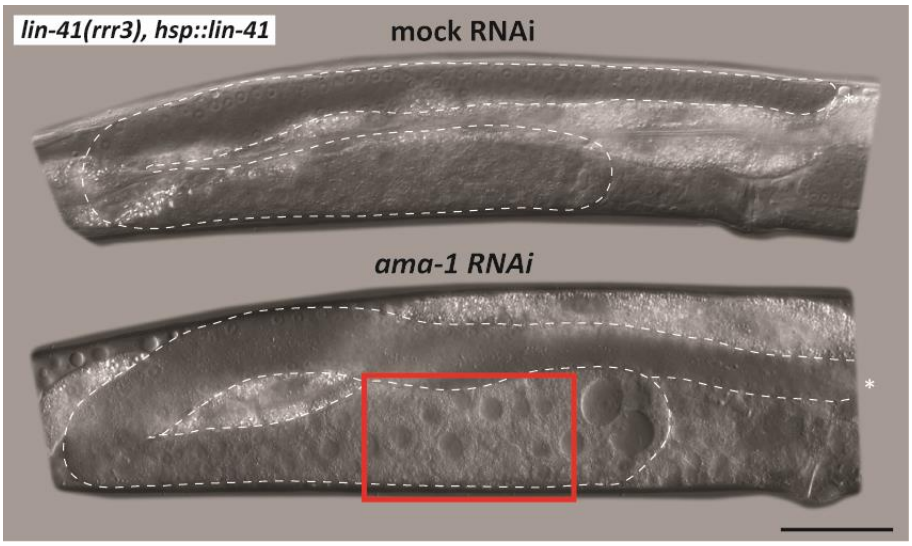


Figure 2.4. Inhibition of Pol II-dependent transcription in teratomous gonads promotes oogenesis: **A.** DIC micrographs of live *lin-41(rrr3)* mutants animals grown at 25°C with rescued somatic phenotype by the *hsp::lin-41* transgene on mock RNAi versus *ama-1 RNAi*. The experiment was started from early L4 to allow germline development until adulthood. 18/18 worms on mock RNAi showed the typical *lin-41* null germline phenotype, whereas 11/14 worms on *ama-1 RNAi* showed at least a partial rescue of oocyte development. Scale bar: 25 µm.

3. GLD-1- and LIN-41-interacting factors play a role in controlling the EGA in the germline

Cristina Tocchini, Sandra Mühlhäusser and Rafal Ciosk

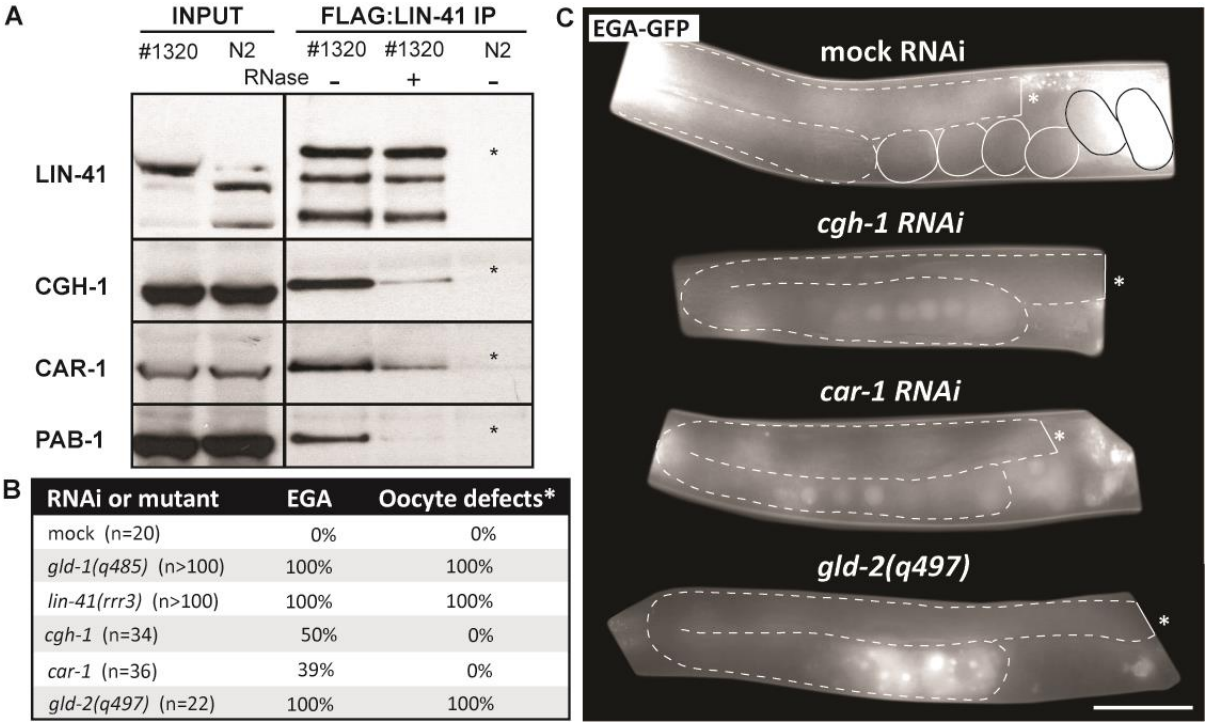
The fact that with our genetic screen we could demonstrate that the EGA can precociously occur in gonads without teratoma formation and that the genes identified are all somehow related to RNA regulation, prompted us to examine whether other RBPs, related to GLD-1, could affect the EGA regulation, as well.

A previous study from our lab (Scheckel et al., 2012) could provide a list of RBPs which could be immunoprecipitated together with GLD-1 (only for CGH-1 the interaction was shown to be RNA-mediated). Interestingly, among them, also LIN-41 could be identified as a poorly associated factor. Even more interesting was the fact that some of the GLD-1-associated factors (CGH-1, CAR-1, PAB-1 and GLD-2) could immunoprecipitate in a RNA-dependent manner with LIN-41, as well (Fig. 3.1A and Spike et al., 2014), suggesting that they are involved in the regulation of the same mRNAs which are bound by the two major regulators of germline pluripotency. CGH-1 is a DEAD-box RNA helicase and CAR-1 is an LSM- and RGG-domain containing protein and both of them are expected to enable decapping-dependent mRNA degradation (Sheth and Parker, 2003). PAB-1 is a poly(A)-binding protein (Mangus et al., 2003) and regulates mRNA metabolism in the germline together with CGH-1 and CAR-1 (Ko et al., 2013). Finally, GLD-2 is the catalytic subunit of a cytoplasmic poly(A) polymerase (PAP), *i.e.*, it lacks the an RNA recognition motif, and regulates gene expression post-transcriptionally (Wang et al., 2002). To test if these factors had, indeed, an impact on the EGA regulation in the germline, we either subjected our EGA-GFP reporter to RNAi (*cgh-1*, *car-1* and *pab-1*) or crossed it into the mutant background (*gld-2(q497)*). Strikingly, we observed precocious onset of the EGA in the germlines of three out of four of the genes we tested (Fig. 3.1B-C). The single knock-down of both *cgh-1* and *car-1* didn't lead to major oocyte defects, but, like the class I mutant from our genetic screen, we could still observe a significant amount of gonads with EGA-GFP-positive oocytes (Fig. 3.1B-C). The *gld-2* mutant, instead, is not able to produce oocytes, but, despite that, the proximal gonads were all EGA-GFP-positive (Fig. 3.1B-C). It is interesting to see how factors associated (in a more or less directed manner) with GLD-1 result to interact, in an RNA-dependent manner, with the other main regulator of pluripotency in the *C. elegans* germline, LIN-41. Although their overall phenotype is not as dramatic as the teratoma formation observed in *gld-1* and *lin-41* null mutants, the fact that knock-down of *cgh-1* and *car-1* or knock-out of *gld-2* determine the precocious onset of the EGA in their gonads strongly suggests an interplay of these

factors with GLD-1 and LIN-41 in controlling the precocious entrance of germ cells into an embryonic state. Similarly to the class I mutants, also down-regulation of *cgh-1* and *car-1* exhibits EGA-GFP-positive oocytes without any other apparent major germline defects: together with *gld-1(rrr1)* and *drh-3(rrr2)*, they could provide an additional source to understand how the very early steps of embryonic transcription are regulated and their mutants could be added to the experiments of RNA-seq and ribosome profiling, suggested for the class I mutants.

As mentioned, the single knock-down of *cgh-1* or *car-1* does not determine the loss of oocyte production: comparing the DIC pictures of mock RNAi versus *cgh-1* or *car-1 RNAi* (Fig. 3.2 – upper panel), it appears clear that, anyway, oocytes are not fully wild-type-looking suggesting that some aspects of their development get compromised. Still, in these RNAi conditions oocytes are able to be formed and, in the case of *car-1* or mild *cgh-1 RNAi*, they can even get efficiently fertilized and give rise to a zygote which will soon after die due to defects in cytokinesis (Audhya et al., 2005). The *lin-41* mutant is known to give rise to teratomous proximal germlines (see attached manuscript, Tocchini et al., 2014). Interestingly, when we tried to RNAi either *cgh-1* (Fig. 3.2 – lower panel) or *car-1* (data not shown) in the *lin-41(rrr3)* mutant background, we observed the very same new phenotype: the germlines were not able to differentiate nor in oocytes (*cgh-1* or *car-1* single RNAi) or somatic cells (*lin-41* single mutant) (Fig. 3.2 – lower panel) and the uncellularized proximal germ cell nuclei appeared to be stacked in the pachytene stage. These data suggest a genetic interaction of *lin-41* with *cgh-1* and *car-1*, which is further supported by an RNA-mediated interaction of the three factors. It is worth mentioning is that the synthetic phenotype observed down-regulating *cgh-1* or *car-1* in the *lin-41(rrr3)* mutant back-ground was already described in other two related mutants: the class B of *gld-1* mutants, in which two different mutations affect the *gld-1* locus (Francis et al., 1995) and the *daz-1* mutant (Karashima et al., 2000; Maruyama et al., 2005), where the DAZ-1 protein is also one of the RBPs which have been shown to be pulled down together with GLD-1 (Scheckel et al., 2012). Not much is known the homolog of the human Deleted in AZoospermia (DAZ), DAZ-1: it is required for the progression from the pachytene stage to the next ones and in the switch from spermatogenesis to oogenesis (Karashima et al., 2000; Otori et al., 2006). Considering the phenotype a question arises: can it be that the lack of DAZ-1 does not lead to teratoma formation simply because it regulates transcripts belonging to different pathways, in a similar manner to what has been shown for its murine homolog DAZL (Chen et al., 2014)? And if this is the case, may it be that it contemporary post-transcriptionally controls both targets of LIN-41 (see next paragraph) and CGH-1/CAR-1? Although it is hard to answer these questions and draw a model out of these few observations, it appears, anyway, clear that there is a tight interaction between all these

factors in regulating oocyte development, not only in promoting it, but also in avoiding the precocious entrance into the next developmental stage, *i.e*., embryogenesis.



B

RNAi or mutant	EGA	Oocyte defects*
mock (n=20)	0%	0%
gld-1(q485) (n>100)	100%	100%
lin-41(rrr3) (n>100)	100%	100%
cgh-1 (n=34)	50%	0%
car-1 (n=36)	39%	0%
gld-2(q497) (n=22)	100%	100%

Figure 3.1. GLD-1- and LIN-41-interacting factors also contribute to control the EGA: **A.** Western blot of FLAG IP. "#1320" represents the strain carrying the transgene coding for the FLAG- and GFP-tagged wild-type LIN-41; N2 is the wild-type strain with no transgene used as a control. CGH-1, CAR-1 and PAB-1 interact with LIN-41 in an RNA-dependent manner. The residual bands for CGH-1 and CAR-1 in the RNase treatment might be explained by a not sufficient amount of RNase used in the experiment. **B.** Quantification of the penetrance for the phenotypes taken into account for the different genotypes. "EGA" stands for the EGA-GFP in the gonads and "Oocyte defects*" for the major oocyte defects (*i.e.*, no oocyte formation). **C.** Fluorescent micrographs of live animals expressing EGA-GFP. Wild-type control has no EGA-GFP in its gonad. *cgh-1* and *car-1* RNAi abnormally express EGA-GFP in their oocytes. *gld-2(q497)* mutant abnormally express EGA-GFP in its proximal gonad which lacks oocytes. Scale bar: 25 µm.

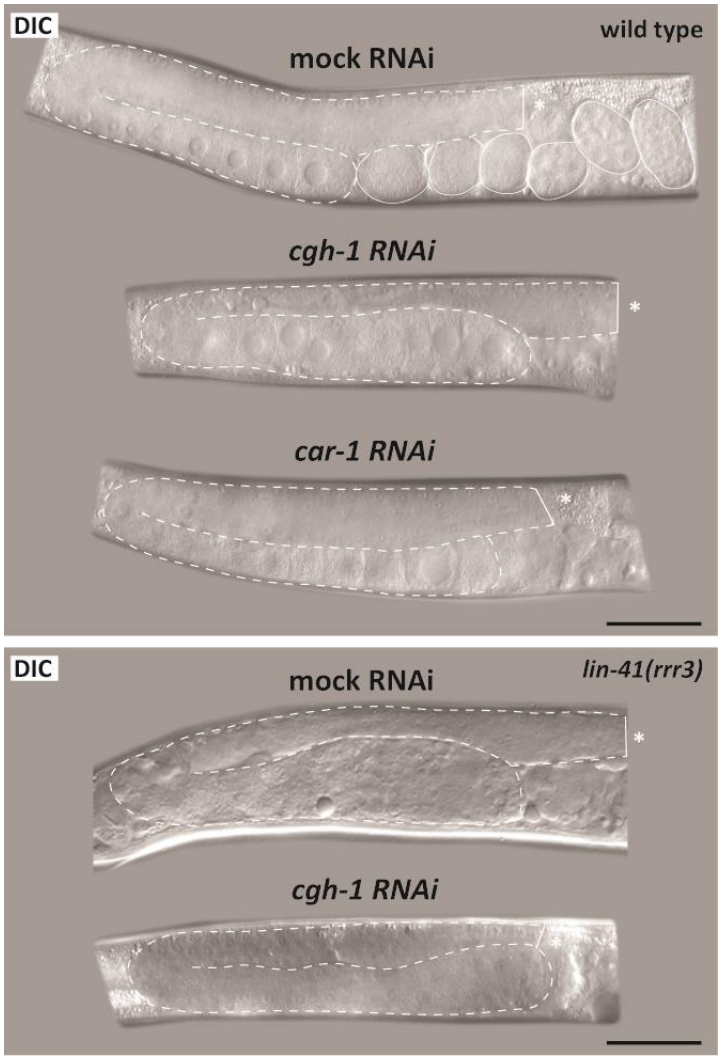


Figure 3.2. *cgh-1* (or *car-1*) *RNAi* in the *lin-41* mutant abckground shows a new phenotype: DIC micrographs of live animals. Upper panel: wild-type animals subjected to mock, *cgh-1* and *car-1 RNAi* (DIC correspondents to the fluorescent micrographs shown in Fig. 3.1C). Lower panel: *lin-41(rrr3)* animals subjected to mock or *cgh-1 RNAi*. Scale bars: 25 µm.

Materials and methods

Additional RNAi clones, mutants and transgenic lines

For RNAi, L1 larvae (early L4 for *ama-1*), grown at 25°C, were fed with bacteria expressing dsRNAs (targeting *cgh-1* and *B0511.6* from the Open Biosystem library or *ama-1* and *car-1* from the Ahringer library) and screened half a day (*ama-1*), one day (*cgh-1* and *car-1*) or one and a half day (*B0511.6*) after the L4-to-adult molt of the same generation. A bacterial strain carrying an "empty" vector was used as a negative control (mock RNAi).

Lab number	**Genotype**
1507	*lin-41(rrr3) (I); rrrSi297[Plin-41::FLAG-GFP-LIN-41(Y941A)::lin-41 3'UTR; unc-119(+)] (II); rrrSi319[Pvet-4::NLS:mCherry:h2b::tbb-2 3'UTR; unc-119(+)] (IV)*
1607	*lin-41(rrr3)/hT2[qIs48] (I;III); rrrSi380[Plin-41::FLAG-GFP-LIN-41(NHLx4)::lin-41 3'UTR; unc-119(+)] (II)*
904	*gld-2(q497)/hT2[qIs48] (I;III); rrrSi198 (IV)*
1093	*gld-1(rrr1)/hT2[qIs48] (I;III); rrrSi199 (II); rrrSi198 (IV)*
1350	*drh-3(rrr2)/hT2[qIs48] (I;III); rrrSi199 (II); rrrSi198 (IV); glo-1() (X)*

Mutating LIN-41

The following primer pairs were used to introduce the point mutations in the BB2 domain:

lin-41_C479S_SDM_Fw

CCTCGAAGCTCTTCTGTGAGTGGAACTCATGATTCAG

lin-41_C479S_SDM_Rv

CTGAATCATGAGTTCCACTCACAGAAGAGCTTCGAGG

lin-41_C490S, C493S_SDM_Fw

CTCATGATTCAGTAATCATTGGAATCAGTGAGAATAGTCCTCATTCAGTTCTATTGTGTGC

lin-41_C490S, C493S_SDM_Rv

GCACACAATAGAACTGAATGAGGACTATTCTCACTGATTCCAATGATTACTGAATCATGAG

lin-41_C500S, C503S_SDM_Fw

GAATTGTCCTCATTCAGTTCTATTGAGTGCAATCAGTGTTGCTCAACATCCTGGAAAGC

lin-41_C500S, C503S_SDM_Rv

GCTTTCCAGGATGTTGAGCAACACTGATTGCACTCAATAGAACTGAATGAGGACAATTC

The following primer pairs were used to introduce the point mutations in the NHL domain:

LIN-41_G886D_SDM_Fw

CAAAGTTCGGAACCAGTGACAATCGTCCAGGACAGTTTG

LIN-41_G886D_SDM_Rv

CAAACTGTCCTGGACGATTGTCACTGGTTCCGAACTTTG

LIN-41_H914L_SDM_Fw

GGTGGCTGATAAAGATAATCTTCGTGTCCAGGTATTCGATG

LIN-41_H914L_SDM_Rv

CATCGAATACCTGGACACGAAGATTATCTTTATCAGCCACC

LIN-41_R991A_SDM_Fw

GAACTTGGACTCGCCAGCTGGATTGTGCTATCTGC

LIN-41_R991A_SDM_Rv

GCAGATAGCACAATCCAGCTGGCGAGTCCAAGTTC

Immunoprecipitation and western blotting

Synchronized worms were harvested as young adults and frozen as worm pellets in liquid nitrogen. Pellets were grinded in mortar under liquid nitrogen to fine powder, resuspended in Extraction Buffer (EB) (50 mM Hepes, 100 mM KOAc, 5 mM MgAc, 0.1 % Triton X-100, 10% Glycerol, 20 mM β-glycerolphosphate, 3 µM Pepstatin A, 3 µM Aprotinin, 1 mM PMSF and Complete protease inhibitor mix (Roche)) and cleared by centrifugation at 20.000 x g for 20 minutes at 4°C. The cleared lysate was incubated with Protein G Dynabeads (Life Technologies) for 2 hours at 4°C on a rotating wheel for pre-clearing. Pre-cleared lysate was subjected to anti-Flag M2 antibodies (Sigma) at 4°C on rotating wheel. When indicated, 1 mg/ml RNase A (Sigma) and 0.5 u/µl RNase T1 (Sigma) was added to the reaction. After 15 hours anti-mouse IgG Dynabeads M-280 (Life Technologies) were added and further incubated for 2 hours at 4°C on rotating wheel. Beads were washed 2 times with EB supplemented with 300 mM NaCl, 2x with EB and 1x with PBS. Bound proteins were eluted by boiling in 1x SDS-Loading buffer (Invitrogen), separated by SDS-PAGE and electro-transferred to PVDF membranes. The following primary antibodies were used: anti-LIN-41 (courtesy of Helge Grosshans, “4796”, 1:200); anti-PAB-1 (Scheckel at al., 2012, 1:50); anti-CAR-1 (Boag et al, 2005, 1:2.500); anti-CGH-1 (Boag et al, 2005, 1:2.000).

Yeast-two-hybrid

The yeast-two-hybrid screen was set up by the company DualsystemsBiotech©. The wild-type isoform B of LIN-41 was used as a bait. Three prays were tested for each protein: the "*Caenorhabditis elegans* whole adult library (P02105)" was used for the prays. The results (putative LIN-41B interactors) were subdivided into three classes: (A) interactors which have been rescued at least three times, representing highly likely interactors; (B) interactors which have been identified twice, representing likely interactors; (C) interactors which have been found only once in the screen ("singletons") and, although some of those may indeed represent true interactors of the protein of interest, others simply represent common false positives.

RNA extractions, microarray and RNA-seq experiments

RNA for the microarray experiment was isolated from fifty gonads dissected from animals one day after the L4-to-adult molt. Two biological replicates were made for each strain (wild-type, *gld-1(rrr1)* and *drh-3(rrr2)*). cDNA was synthesized with oligo(dT) primers using the ImProm II Reverse transcription system from Promega according to manufacturer's instructions. cDNA from the two biological replicates of each strain was used for tiling array analyses.

For RNA extraction and RNA-seq experiment were performed as previously described (Arnold et al., 2014). Animals were grown at 25°C and harvested half a day after the L4-to-adult molt.

Statistical analyses

Statistical analyses and plots were generated with the use of the statistical software R: version 2.12.2 (2011-02-25). Copyright © 2011 The R Foundation for Statistical Computing. ISBN 3-900051-07-0. Platform: i386-pc-mingw32/i386 (32-bit). For the identification of common motives among the promoters of the E.E. genes, we used the software for motif discovery HOMER (v4.7, 8-25-2014) (Heinz et al., 2010).

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