Molecular and Developmental Characterization of Functions of the *let-7* miRNA and its Target LIN-41 in Controlling Temporal Patterning in *C. elegans*

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2. Summary

Multicellular organisms start their life as a single cell. During the development of an organism, this single cell proliferates and differentiates forming specialized cells such as neurons, muscle cells, epithelial cells and germ cells. Amazingly, each of these unique cells although having specialized functions, contains an identical genome that is regulated selectively to express a particular set of genes that are specifically required to create a certain cell type. This selective gene expression is the basic principle through which complex multicellular organisms such as humans are formed by repeating two fundamental processes, cell proliferation and cell specialization or differentiation. The regulation of gene expression to increase or decrease the production of specific gene products (protein or RNA) can be modulated at various levels, from transcriptional initiation, to post-transcriptional RNA regulation, and to the post-translational modification of a protein. Mis-regulation at any of these levels perturb the gene expression, thus resulting in disease state

This thesis delves into elucidating how the *lethal-7 (let-7)* miRNA and its target *lineage defective 41 (lin-41)* function as post-transcriptional regulators of gene expression. Specifically, I investigated how these genes temporally control gene expression to specify the timing of developmental events in well studied developmental pathway also known as the heterochronic pathway in *Caenorhabditis elegans* worm.

In the first part of this thesis I attempted to uncover a direct interface between the *let-7* miRNA and the cell cycle machinery. The goal of this project was to assess how *let-7* regulates cell cycle exit and terminal differentiation by regulating target genes directly by binding to their respective 3'UTRs. We performed an RNAi screen against 40 core cell-cycle regulating genes for *let-7* lethality suppression and demonstrated that RNAi against two genes, *cdk-1* and *cdc-25.2*, not only suppressed the *let-7* lethality, but also reversed the retarded seam-cell phenotypes, i.e. additional seam cell divisions and alae defects, in *let-7(n2853ts)* as well as *let-7(mn112)* animals, confirming specificity of the genetic interaction. However, detailed analysis revealed that although the 3'UTRs of these two mitotic genes might confer post-transcriptional repression, this seems unlikely to be a consequence of *let-7* function. Furthermore, we also examined *cdk-1::gfp* expression and found that knock-down of *lin-29,* which is downstream of *let-7*, resulted in elevated levels similar to the effect of *let-7* knock-down. Similarly, up-regulation was also observed for RNAi of *mab-10*, a transcription co-factor that acts in concert with LIN-29 to promote differentiation of the hypodermis. Thus, we conclude

that *let-7* preferentially regulates *cdk-1* indirectly, in a manner that requires the LIN-29 transcription factor.

For the second part of this thesis, I focused on *lin-41,* which is a direct target of *let-7*. The project involved functional characterization of LIN-41. Specifically in this project we attempted to address two important questions namely how does LIN-41 protein mechanistically regulate gene expression post-transcriptionally and what are its targets.

Towards that goal we created a worm strain expressing a functional tagged version of LIN-41. Using the tagged LIN-41, we established its mRNA binding potential by performing LIN-41 coimmunoprecipitation and assessing the mRNA targets of LIN-41 getting pulled down. Our analysis revealed multiple mRNAs i.e. *lin-29, mab-10, dmd-3* and *mab-3* that associated with the LIN-41 protein. For *lin-29* mRNA, we also tested where on the mRNA would LIN-41 protein bind. Our analysis revealed that LIN-41 binds in the 5'UTR region in the *lin-29* mRNA and not the 3'UTR region. Additionally our analysis also revealed that the regulation of *lin-29* happens at the translational level, by LIN-41 protein binding to *lin-29* mRNA in the 5'UTR region.

By analyzing worm strains with mutations in the individual domains of LIN-41 we obtained further insight into how LIN-41 may carry out its role as a post-transcriptional regulator of gene expression especially in the context of the somatic tissue development. Thus, we found the NHL domain to be critical for the somatic function of LIN-41. Finally, we tried to identify protein-binding partners of LIN-41 that could play an important role in LIN-41-mediated post-transcriptional gene regulation. However, it remains to be established if the putative protein partners that we found in our analysis are true interaction partners of LIN-41, and if so, how they participate in LIN-41's functions.

Taken together, this work has provided further insight into the functioning of two post-transcriptional regulators of gene expression**,** *let-7* miRNA and its downstream target *lin-41*.

3. Introduction

3.1. Regulation of gene expression controls developmental programs in an organism

In the cell, regulation of gene expression modulates the rate of production of a specific gene product that can be either protein or RNA in response to external stimuli or to trigger a developmental pathway. During the process of gene expression, DNA is transcribed into messenger RNA (mRNA), which transfers the genetic information by serving as a template for protein synthesis (Crick 1970). In eukaryotes the process of gene expression begins in the nucleus, where gene-specific transcription factors bind to the promoter region of a gene, thus initiating the transcription process. Regulating gene expression at the transcription level has been considered as the most extensively used control point, which not only alters the number of mRNA molecules that are transcribed but also determines when the gene is transcribed (Clancy and Brown 2008; Levine and Tjian, 2003). However, recent studies have revealed that in addition to transcription regulation, eukaryotes have evolved multiple mechanisms in order to enable more intricate control over regulation of gene expression (Day and Tuite 1998). These additional levels of regulation occur on the messenger RNAs as well as on the proteins in order to modulate gene activity and collectively, they are referred to as post-transcriptional regulation of gene expression (Moore 2005).

3.2. RNA metabolism is a major access point to regulate gene expression post-transcriptionally

RNA metabolism refers to any event in the life cycle of ribonucleic acid (RNA) molecules, including their synthesis, folding/unfolding, modification, processing and degradation. RNA metabolism plays a very important role in regulating gene expression (Yost et al., 1990). Current research has revealed that an increasingly important level at which the RNA regulation occurs is the post-transcriptional level, thus enabling cells to provide a rapid response mechanism to modulate RNA levels and protein synthesis, since regulation of gene expression through transcriptional initiation may require hours to take effect (Sharp 2009; Halbeisen et al., 2008).

In eukaryotes, an RNA molecule is transcribed from DNA in the nucleus as pre-mRNA. These pre-mRNAs undergo several processing steps that typically include capping, pre-mRNA splicing, and polyadenylation to generate the final messenger RNA (Moore and Proudfoot 2009). mRNAs are then

transported to the cytoplasm where they are either stored or recruited by the protein synthesis machinery to initiate translation and then finally degraded (Clancy and Brown 2008). Each of these steps is subjected to regulation leading to fine-tuning of gene expression in some cases, where as in others, regulation of gene expression is entirely carried out post-transcriptionally (Moore 2005; Halbeisen et al., 2008; Garneau et al., 2007).

RNA metabolism directly affects protein production by modulating the efficiency of translation process. During translation the mRNA serves as a template to assemble a chain of amino acid monomers that form the basic building blocks of the protein. These amino acids are linked by peptide bonds that are catalyzed by peptidyl transferase activity of a large RNP complex known as the ribosome, which consists of proteins, ribosomal RNA and transfer RNA (Cech 2000 & Jackson et al., 2010). Translation can be divided into three steps: translation initiation, elongation and termination. Usually the translational regulation occurs at initiation step under most conditions, where the recruitment of ribosomes on the transcribed mRNA is controlled. This allows rapid generation of proteins when a signal activates translation (Jackson et al., 2010).

3.3. 5’ and 3’ untranslated regions of mRNAs play crucial roles in regulation of mRNA transcript stability and translation

Although the primary method of gene expression modulation happens at the transcription level, recent studies have revealed that many mRNA transcripts in eukaryotic cells have long half-lives of >6 hours, which led researchers to believe that the regulation of gene expression at the level of translational control plays an increasingly important role in eukaryotes (Raghavan et al., 2002).

Furthermore, sequencing of the human genome led to an in-depth understanding of human gene structure. Recent study has found that for an average size of a human gene of 50kb, less than 2% appeared to code for proteins, whereas the remaining 98% was noncoding region consisting of the introns and the 5’ and 3’ untranslated (UTR) regions (Lander et al., 2001). Although these noncoding sequences are transcribed, they are never translated and recent reports have suggested that the non-coding sequences apparently play a critical role in modulating gene activity at the post-transcriptional level (Barrett et al., 2012).

Although many features of an mRNA can contribute to its translation, most control elements are located within the untranslated regions. The 5’ m7GpppG cap and the 3’ poly(A) tail are not only important determinants of

translational efficiency but also mRNA stability/decay rate (Barrett et al., 2012; Day and Tuite 1998; Coller and Parker 2005). mRNAs are protected by the presence of 5' 7-methylguanosine cap structure, which prevents mRNA degradation and facilitates translation initiation (Shatkin and Manley 2000). A 3' poly(A) tail protects the mRNA from 3'-to-5' exonuclease-based degradation and also promotes translation initiation (Munroe & Jacobson 1990). Furthermore, the presence of a 5' UTR containing regulatory elements consisting of secondary structure such as the stem-loops also negatively affect translation by impeding the binding or migration of 40S ribosomal subunits. Start site consensus sequence, upstream open reading frames (uORFs) or AUGs, terminal oligo-pyrimidine (TOP), and internal ribosomal entry sites (IRES) are all motifs within an mRNA that can determine translational efficiency (Day and Tuite 1998; Wilkie et al., 2003). Moreover in certain cases, the 5' UTR may contain structured binding sites for regulatory proteins. These 5' UTR-interacting proteins are known to repress translation (Barrett et al., 2012). One such example of repression by a 5'UTR binding protein is the Iron regulatory proteins (IRPs) controlling translation of an mRNA that contain a stem–loop structure known as the iron-responsive element (IRE), in response to intracellular iron concentrations. When there is intracellular iron depletion, IRPs function as RNA-binding proteins that bind IREs with high affinity. The binding of an IRP to the ferritin IRE blocks the association of the 43S translation preinitiation complex with the mRNA and prevents the recruitment of the small ribosomal subunit, thereby repressing the translation of the ferritin protein. Thus, translational regulation by IRPs allow for rapid and coordinated control of proteins that are crucial for regulation of iron levels in cells (Cazzola and Skoda 2000).

The 3'UTR region is also known to contain numerous binding sites for trans-acting regulatory factors. In addition to the protein binding factors, the 3'UTRs also contain sites for binding to a large class of regulatory RNAs that in most cases negatively regulate the expression of a gene by destruction of its mRNA transcript in addition to affecting its translation (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001; Barrett et al., 2012). An example of a trans-acting RNA binding protein is Cytoplasmic polyadenylation element binding protein (CPEB), which binds to the cytoplasmic polyadenylation elements (CPE) present in the 3'UTR of cyclin B1, resulting in translational repression (Hake and Richter, 1994). Another example of a positive regulation by the elements present in the 3'UTR region is of the transferrin mRNA. Transferrin contains IREs within its 3′UTR that mediate positive translational activation by interacting with the IRP. Such interactions stabilize the stem loop structure, thereby increasing mRNA stability and translation (Khosrow et al., 2011; Day and Tuite 1998). In the case of trans-acting RNA exerting regulation, the best example is of the two miRNAs *lin-4* and *let-7*, which bind to the 3'UTR region of their targets, thus mediating

developmental regulation in *C. elegans* worm (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). Furthermore, 3 'UTR region may also contain localization elements or zipcodes, which allow the mRNAs to localize to specific compartments in the cytoplasm of the cell (Khosrow et al., 2011).

3.4. Role of mRNA localization and RNA granules in post-transcriptional gene regulation

In many cases the mRNA transcripts in the cytoplasm of a eukaryotic cell associate with one of various cytoplasmic RNA granules (Anderson and Kedersha 2006). These RNA granules have emerged as important players in the post-transcriptional regulation of gene expression (Keene 2001; Khosrow et al., 2011). These granules are composed of a large number of protein components, which include RNA binding proteins, RNA helicases, decay enzymes, scaffold proteins and translational factors (Angenstein et al., 2005). In fact these cytoplasmic RNA granules have been proposed as the primary sites of post-transcriptional gene regulation, by controlling localization, stability, and translation of their associated mRNAs (Anderson and Kedersha 2009; Besse and Ephrussi 2008). The cytoplasmic granules can be divided into four groups: germinal granules, stress granules, processing bodies (P bodies), and neural granules.

Germinal granules as the name suggests are found in germline of an animal and are well studied in the metazoan germlines (Jud et al., 2008; Gallo et al., 2008; Pitt et al., 2000; Anderson and Kedersha 2009). These RNA granules play an important role in gametogenesis and embryonic development (Noble et al., 2008). They contain proteins that are involved in translation initiation, translation control, and mRNA decay and are proposed in regulating maternal mRNA expression. Some of the proteins that are found in germinal granules include CAR-1, which is an Sm protein related to Lsm proteins that regulate mRNA splicing, decapping, and decay (Squirrel et al., 2006). These granules also contain a dead box RNA helicase CGH-1 related to Dhh1 and p54/Rck, enzymes, which is involved in translational silencing and decapping as well as decapping enzyme DCP1 and orthologues of the translation initiation factors eIF4E and eIF5A (Rajyaguru and Parker, 2009; Boag et al., 2008; Navarro et al., 2001).

Granules that are formed, when the cell encounters stresses due to depletion of transcription factors, impaired translation initiation, and other conditions are called stress granules (Anderson and Kedersha 2009). They are dynamic cytoplasmic foci composed of non-translating mRNAs and associated RNA binding proteins, translation initiation proteins, the small ribosomal subunit

(40S) and many of the stress response proteins such as the heat shock proteins (Anderson and Kedersha 2008; Anderson and Kedersha 2009; Khosrow 2011).

Similar to the stress granules is another class of RNA-protein foci called P-bodies (Sheth and Parker 2003; Eulalio et al., 2007). There is a positive correlation between formation of P-bodies and its association with untranslated mRNAs. P-bodies are found to contain various mRNA decay machinery such as CCR4-NOT1 complex, decapping enzymes Dcp1/Dcp2, RCK/p54, Pat1, Scd6/RAP55, Xrn1 exonuclease and miRNA repression machinery. P-bodies also have been suggested to play a role in mRNA decay, although conclusive evidence is still missing (Parker and Sheth 2007).

Another type of RNA granules typically found in neurons are the neuronal granules (Anderson and Kedersha 2009). In order to regulate protein expression in the distant synaptic region in the neuronal cells, a distinct process has evolved, involving packing mRNAs with associated proteins and transporting the mRNAs in the form of neuronal granules. Neuronal granules are involved in translational silencing of the mRNAs until needed and release the mRNAs for translation upon specific stimuli (Rodriguez et al., 2008; Anderson and Kedersha 2009).

Thus mRNA compartmentalization in the form of RNA granules allows a cell to control protein synthesis in response to specific stimuli without the need for gene transcription.

3.5. RNA binding proteins and non-coding RNAs play critical role in post-transcriptional gene regulation

A large group of regulators consisting of RNA binding proteins controls the fate of mRNA during its life cycle. These RNA binding proteins influence multiple aspects of RNA metabolism such as RNA processing, localization, storage, degradation, and translation efficiency, thus playing an essential role in post-transcriptional gene expression to regulate cellular phenotype (Glisovic et al., 2008). These RNA binding proteins carry out their function by targeting specific subsets of pre-mRNA/mRNA transcripts by typically interacting with the non-coding regions found in the RNA molecule such as the introns and the 5’ or 3’ untranslated regions (UTRs) (Khosrow 2011; Barret et al., 2012). In the cytoplasm these RNA binding proteins influence translational efficiency and mRNA stability by targeting 5′ or 3′ untranslated regions (UTRs) of mRNAs, thus directly influencing protein expression (Wilkie et al., 2003).

A eukaryotic genome codes for greater than 500 RBPs each having unique RNA as well as protein binding affinities and it has been observed that during evolution the increase in the number of introns has coincided with the corresponding increase in the diversity of RNA binding proteins (Castello et al., 2012; Hogan et al., 2008; Anantharaman et al., 2002). A characteristic feature observed in many RNA binding proteins is their modular architecture consisting of multiple repeats of RNA binding domains. Furthermore, many RNA binding proteins also contain associated auxiliary domains that help modulate the activity of these proteins (Burd and Dreyfuss 1994). Till date many RNA binding domains have been identified. These include the RNA-recognition motif- (RRM), which by far the most common RNA-binding protein module, the K-homology (KH) domain, the dsRBD domain, RGG box, Sm domain, DEAD/DEAH box and RNA-binding zinc-finger (ZnF) domains (Chen and Varani 2005; Lunde et al., 2007). Surprisingly, recent studies of mRNA-bound proteome in yeast and mammalian cells have demonstrated that many proteins without canonical RNA binding domains can also bind RNA (Baltz et al., 2012; Castello et al., 2012; Klass et al., 2013; Tsvetanova et al., 2010), thus signifying how limited our understanding is about these RNA binding proteins and also their function (Glisovic et al., 2008). An example of an RNA binding protein, which not only controls mRNA localization but also regulates translation, is the ZBP1. ZBP1 contains four KH domains and one RBD. The protein binds to β-actin mRNA in the nucleus via 54 nucleotide long element in the 3′ UTR of β-actin known as the zipcode, which results in the transport of the mRNA into the cytoplasm. In addition to β-actin localization, ZBP1 also regulates the translation of β-actin mRNA by blocking translation initiation (Oleynikov and Singer 2003; Ross et al., 1997).

In addition to the RNA binding proteins a new class of regulatory RNAs have been recently shown to carry out regulation by binding to the mRNA in a sequence specific manner. One class of small noncoding RNAs that are 22 nucleotides in length known as microRNAs (miRNAs) negatively regulate target gene expression at the post-transcriptional level by binding to the 3' untranslated region of an mRNA with partial complementarity (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). miRNA function by serving as a guide molecule to bring the miRNA induced silencing complex (miRISC) to the 3'UTR of the target gene (Meister and Tuschl 2004, Tomari and Zamore 2005; Buchan and Parker 2007; Eulalio et al., 2008). As discussed in later section, *let-7* is one such example of a miRNA, which plays a critical role in the development of *C. elegans* through post-transcriptional gene regulation of its target *lin-41* (Rheinhart et al., 2000; Slack et al., 2000). Recent study has revealed the molecular mechanisms behind the post-transcriptional regulation of target gene by miRNA to not only involve translational repression and but also degradation of target mRNAs with the help of various effector proteins present in the miRISC complex such as

CCR4-NOT deadenylase complex, AGO, GW182, PAN2-PAN3 (Fabian et al., 2010; Fabian et al., 2009; Braun et al., 2011; Chekulaeva et al., 2011; Mathys et al., 2014, Chen et al., 2014).

Collectively the post-transcriptional mechanisms have been increasingly recognized as key regulators of gene expression.

3.6. *Caenorhabditis elegans* (*C. elegans*)

C. elegans is a microscopic, free-living soil nematode (roundworm) that is found in the temperate climates and feeds primarily on bacteria. *C. elegans* is frequently used in biological research and has been a popular model organism due to its short life cycle, compact genome, small size (1mm) and stereotypical development. An adult worm has a simple body plan and an invariant cell lineage containing 959 somatic nuclei thus enabling cell lineage tracking with relative ease (Brenner. 1974; Byerly et al. 1976; Sulston et al. 1983). These worms can be easily maintained by growing them on agar plates or in liquid culture by feeding them with E. coli bacteria. Due to their transparent nature, *C. elegans* can be easily examined at the cellular level using differential interference contrast microscopy. Moreover, the position of cells is constant, as is the cell number, making it is easy to track cells and follow cell lineages thus providing a great tool for research on how genes influence cell fate. These traits enable the study of the biology of a single cell in an intact, living organism. The *C. elegans* life cycle comprises the embryonic stage, four larval stages (L1-L4) and an adult stage. The end of each larval stage is marked by a molt, during which a new stage specific cuticle is synthesized and the old one is shed. Furthermore, *C. elegans* proved to be an excellent organism to not only perform an efficient forward genetics screen but also RNA-mediated interference (RNAi) by feeding is an easy and rapid technique for targeted gene inactivation. All these properties have enabled *C. elegans* to become a popular model organism for studying multiple aspects of genomics, neuroscience and cell biology.

3.7. *C. elegans* hypodermis is an excellent tissue to study developmental timing event

The outer body wall in *C. elegans* consists of an epidermal cell layer, which is also referred to as hypodermis. The main function of the hypodermis is the secretion of the cuticle, a collagenous structure that protects the animal from the environment and serves as a stable but very flexible exoskeleton (Singh and Sulston 1978; White 1988; Kramer 1997; Yochem et al., 1999). Embedded within the hyp7 cell are subset of hypodermal cells also called as lateral hypodermal blast cells or seam cells (Sulston et al. 1983, Podbilewicz and White 1994). The seam cells are arranged in longitudinal rows on the left and right side of the body, having a smooth tapered cell body. Seam cells are linked to hypodermis by small adherens junction along their apical borders and by small gap junctions on their lateral membranes. Hatched worms contain 10 seam cells on each side of the worm (H0-H2, V1-V6 and T). During worm development, except for H0, these seam cells divide in a stem-cell like pattern before each molt. Between L2 and L4 stages, most of the seam cells (V1-V4 and V6) divisions generate an anterior daughter cell, which fuses with hyp 7 and stops dividing, whereas the posterior daughter cell maintains a stem cell-like fate and continues to divide until the last molt between L4 stage and adult stage. Before final molt, the seam cells exit cell cycle, terminally differentiate and fuse to each other making a longitudinal syncytium containing 16 seam cell nuclei (Sulston and Horvitz 1977).

Seam cells synthesize collagen proteins, which are necessary for the formation of the stage-specific cuticle (Thein et al., 2003). Furthermore, during exit from cell cycle and their final differentiation, seam cells also secrete a set of raised cuticular ridges known as the alae (Sulston and Horvitz, 1977). Thus adult alae formation is used as an indicator of seam cell terminal differentiation. Moreover, seam-cell divisions also generate neurons and glia. For example during L1 stage H2.aa transforms into an anterior deirid cell. Seam lineages also give rise to neurons (PVW and PVN), tail spike neuron (PHC) and support cells of the phasmid (Sulston and Horvitz, 1977). In male worms seam cells are responsible for the formation of sensory rays (Waring and Kenyon 1990).

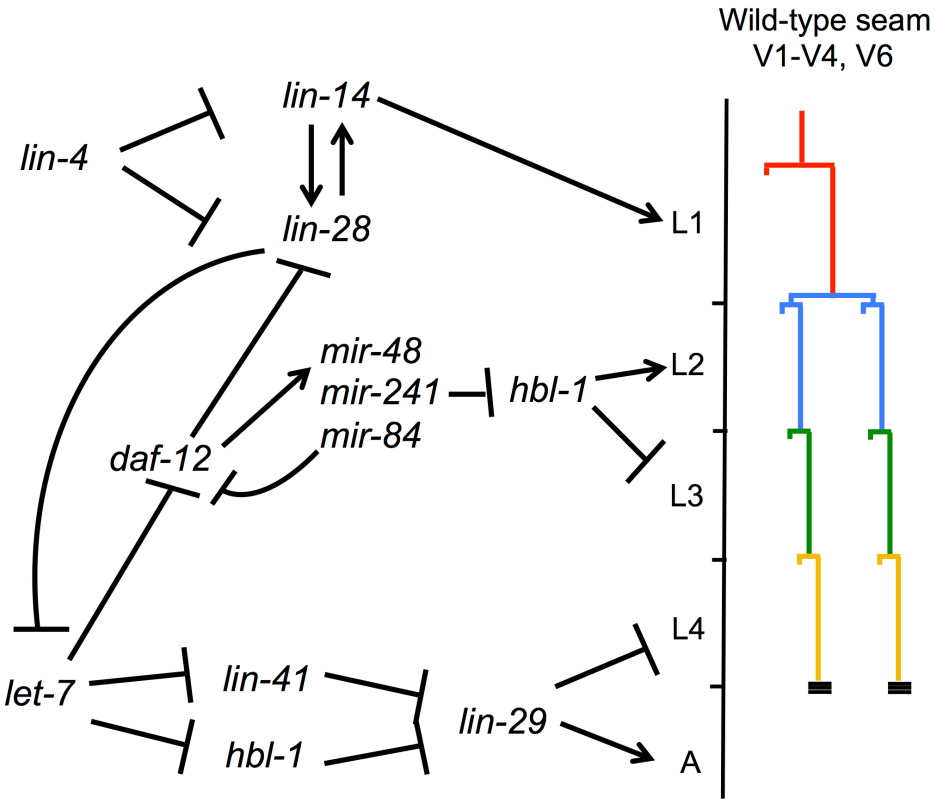


Figure 1. Heterochronic pathway in *C. elegans*
A simplified model of the key effectors in the heterochronic pathway affecting hypodermal development. Arrows indicate activation; bars indicate repression. Regulatory relationships shown here are supported by genetic data. Modified from (Resnick et al., 2010)

3.8. The heterochronic pathway regulates the hypodermal development

In *C. elegans*, the heterochronic pathway consists of a class of genes that control temporal cell fates by regulating the timing of cell proliferation and differentiation events. This pathway plays a critical role in regulating the postembryonic developmental events in the worm by controlling multiple events, such as stage-specific patterns of cell division (Ambros and Horvitz, 1984), progression through the cell division cycle (Ambros and Horvitz, 1984), adult-specific terminal differentiation of hypodermal cells (Ambros, 1989) and dauer larva developmental arrest (Liu and Ambros, 1989).

The regulation of heterochronic pathway genes is critical for proper hypodermal seam cell divisions during worm development. Mutations in these heterochronic genes result in seam cells displaying wrong cell fate of earlier

or later worm stage, leading to precocious or retarded seam cell phenotype (Sulston and Horvitz 1981). One such example is the mutation of *lin-4,* resulting in reiteration of larval stage 1 seam cell division pattern in later stages, thereby failing to produce any adult specific cuticle (Horvitz and Sulston 1980; Chalfie et al., 1981). On the other hand mutant worms for *lin-14* and *lin-28* result in opposite phenotype, displaying precocious cell cycle exit and differentiation in seam cells due to the skipping of larval stage 1 and 2 fates respectively (Ambros and Horvitz, 1984). During the L4 molt, seam cells undergo a final round of cell division, before they permanently exit cell division cycle and terminally differentiate thus marking larval-to-adult transition. Reinhart and colleagues showed that this final transition was regulated by *let-7* (*lethal 7*) gene, which encodes for a micro RNA (miRNA). Seam cells in *let-7* mutant animals fail to exit cell cycle, thus reiterating the L4 cell division in adult stage. Moreover, these cells fail to express adult specific collagens and remain in undifferentiated state (Reinhart et al., 2000).

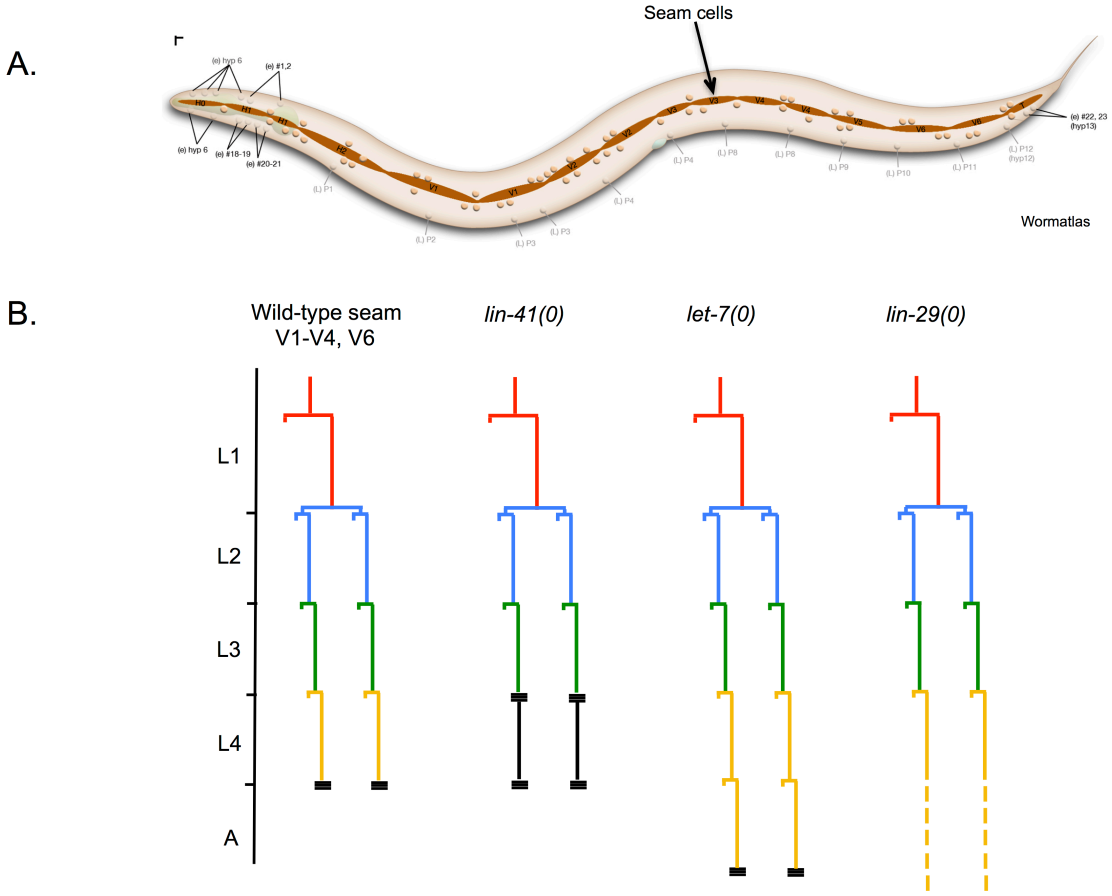


Figure 2. *C. elegans* seam cell development

A. Schematic view of an adult *C. elegans* worm showing seam cells in the center in orange color (arrow) B. Postembryonic cell lineage of the V1-4 and V6 cells in wild-type animals. Seam cells divide during each larval molt, until larval-to-adult transition, when they exit cell cycle and differentiate. *lin-41 (lf)* results in precocious exit of cell cycle as well as differentiation. In contrast *let-7 (lf)* and *lin-29 (lf)* result in over proliferation of seam cells in addition to lack of differentiation. (Modified from Wormatlas and Rougvie 2001)

3.9. *C. elegans* worm is an excellent model organism to study cell cycle regulation

Cell division is a complex process that is regulated through multiple molecular pathways and checkpoints, involving numerous regulatory proteins, which direct the cell through a specific sequence of events resulting in the production of two daughter cells. Such precise control through multiple checkpoints is essential for the regulation of cell cycle, since any perturbation in cell cycle pathway results in cell over proliferation, which is essentially the root cause of diseases such as tumors and cancer (Vermeulen et al., 2003).

The basic mechanism behind cell cycle is largely similar in most eukaryotes. Cell cycle consists of four distinct phases G1, S, G2 and finally the M phase (Norbury and Nurse 1992). In G1 phase the cell prepares itself before committing to cell replication by increasing its size and ensures that all conditions are met before DNA synthesis. Next phase is the S phase where the cell actually carries out the DNA replication. In the G2 phase, cells ensure that DNA has been replicated faithfully. Finally in the M phase the cell stops growing and focuses entirely on orderly division forming the two daughter cells. There are three checkpoints during which the cell ensures that favorable conditions are met before moving into the next phase in the cell cycle. The G1 checkpoint, also known as the restriction checkpoint; the G2/M checkpoint; and the metaphase checkpoint, also known as the spindle checkpoint (Pardee 1974; Hartwell & Weinert 1989). Regulating these checkpoints is a family of protein kinases known as cyclin dependent serine/threonine protein kinases (CDK) that require association with a cyclin subunit for their activation (Nigg 1995). Specific cyclin-CDK complex trigger different phases in the cell cycle by activating or inhibiting different downstream targets thus promoting or halting the cell cycle progression (Harishama et al., 2013; Schafer 1998; Mitchison 1971; Murray and Hunt 1993).

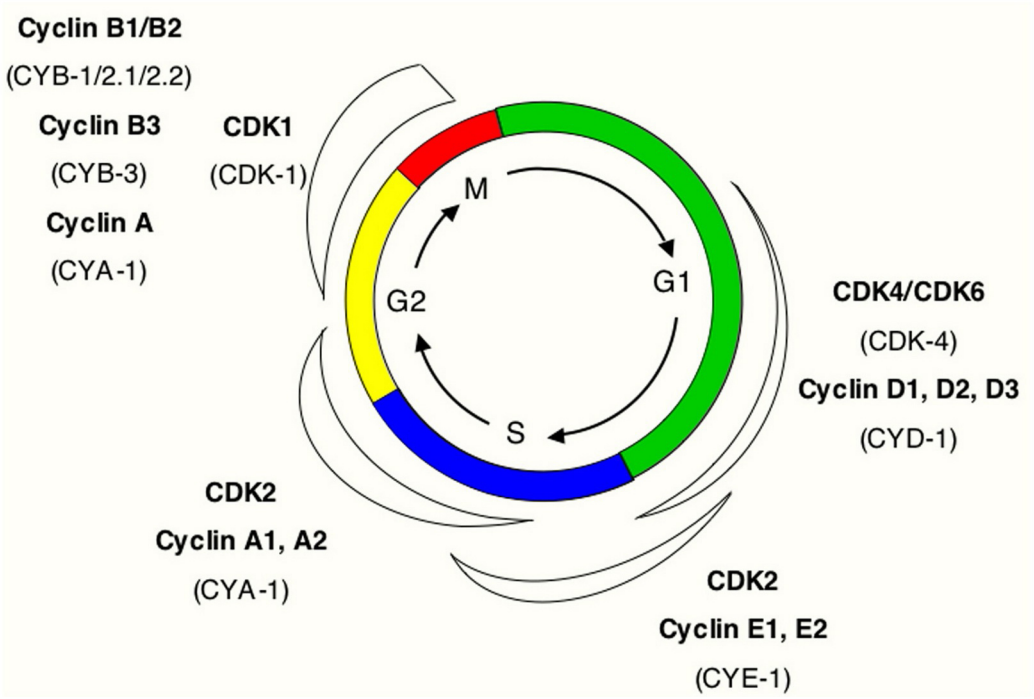


Figure 3. **Cell cycle pathway in *C. elegans***
An approximate time of activity for different combinations of cyclins and CDKs, based on studies of mammalian cyclins and CDKs. *C. elegans* family members are indicated between brackets (Image taken from Wormatlas)

Although cell cycle regulation has been well studied in unicellular organisms such as yeast as well as in cancer cells *in vitro* (Harishama et al., 2013), there is a limited understanding of its regulation in context of development of a multicellular organism.

For proper animal development, it is critical to regulate cell proliferation and cell differentiation by regulating the levels of cell cycle factors. *C. elegans* is a particularly well-suited model to study coordination of cell proliferation and differentiation processes during development due to the fact that the somatic cells undergo cell division at specific times, which is regulated by specific developmental program, thus resulting in largely invariant cell lineage (Sulston et al., 1983; Heuvel and Kipreos 2012; Kipreos 2005). In addition to advantages such as simple body organization, many of the regulators and pathways controlling cell proliferation and cell fate are conserved in higher organisms (Boxem et al., 1999; Boxem and Heuvel, 2001; Park and Krause, 1999).

Furthermore, in terms of cell cycle machinery, in yeast a single CDK (Cdc28p in Saccharomyces cerevisiae and Cdc2 in Schizosaccharomyces pombe) acts with different cyclins to promote progression through G1, S and G2/M (Mendenhall and Hodge, 1998; Moser and Russell, 2000). However, *C. elegans* cell cycle use not only a variety of cyclins but also multiple catalytic subunits, which is similar to their mammalian counter part where specific CDKs act at distinct times in the cell cycle and use specific cyclin partners to promote progression through G_1, S and G_2/M phases (Kipreos 2005; Heuvel and Kipreos 2012; Koreth and Heuvel 2005). In addition to the periodic synthesis and degradation of cyclins, which are the positive regulatory subunits, fluctuations in levels of negative regulators such as CKIs (CDK Inhibitors) (Hong et al., 1998) as well as phosphorylation/dephosphorylation of cell cycle factors exert regulatory effect and are critical for proper progression of cell cycle pathway (Fukuyama et al., 2003). *C. elegans* also contains pathways that are absent in single cell eukaryotes such as Rb/E2F pathway that regulates G1 phase entry (Frolov and Dyson 2004; Boxem & van den Heuvel 2001).

To summarize, *C. elegans* provides an excellent system to put cell cycle in context of an animal development and to study the effects of regulation as well as misregulation of cell cycle during the development.

3.10. *let-7* miRNA is a negative regulator of cell proliferation and functions as pro-differentiation gene

let-7 was originally discovered in *C. elegans* and was identified as one of the key heterochronic gene involved in larval-to-adult (L/A) transition (Reinhart et al., 2000). *let-7* belongs to a class of small noncoding RNAs that are 22 nucleotides in length and are known as microRNAs. Several studies have revealed that *let-7* miRNA functions as a key regulator of development and that it is highly conserved across species as diverse as nematodes to humans, suggesting that *let-7* is an evolutionarily ancient gene (Pasquinelli et al., 2000). In addition to the sequence conservation, some of *let-7* miRNA's targets as well as its function are highly conserved across different species (Grosshans et al., 2005; Johnson et al., 2005; Lin et al., 2007; Kanamoto et al., 2006 ; O'Farrell et al., 2008; Roush and Slack 2008).

Various studies carried out over the past few years have revealed several *let-7* targets, thus starting to provide an insight about how *let-7* may function in regulating the development and how its mis-regulation results in the disease of an animal (Rheinhart et al., 2000; Slack et al., 2000; Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros 2001; Banerjee and Slack 2002). One example of a well-studied and highly conserved interaction is between *let-7*

and its target *lin-41* (O'Farrell et al., 2008; Kloosterman et al., 2004; Kanamoto et al., 2006; Rybak et al., 2009; Lin et al., 2007; Slack et al., 2000). In subsequent years, additional interaction partners of *let-7* were discovered. For example in one study it was revealed that *let-7* negatively regulates one of the well-established pluripotency factor *lin-28*, which is required to maintain stemness (Moss and Tang, 2003). Moreover, *let-7*'s interaction with multiple oncogenes such as *let-60* in *C. elegans* and its human homolog RAS (Johnson et al., 2005), MYC and HMGA2 (Lee and Dutta. 2007) suggested that this miRNA plays a critical role as a tumor suppressor. Indeed many tumors and cancers showed reduced expression of *let-7* miRNA resulting in loss of regulation over critical factors required to control cell proliferation and differentiation (Bussing et al., 2008). The loss of *let-7* family members also has prognostic value as it indicates poor survival rate in cancer patients (Barh et al., 2010). For example down-regulation of *let-7* was found to correlate with poor survival in lung cancer (Takamizawa et al., 2004; Esquela-Kerscher et al., 2008; Kumar et al., 2008). A combined loss of *let-7*d with an increase in expression of the *let-7* target high mobility group 2A (HMGA2) was indicative of poor survival in ovarian cancer (Shell et al., 2007).

In addition to *let-7*'s role in disease, this miRNA plays a critical role in normal development of an organism. For example in Drosophila, *let-7* mutants display a temporal delay in the terminal cell cycle exit in the wing and also have defects in maturation of neuromuscular junctions in adult abdominal muscles. The mutants exhibit clear juvenile features in their neuromusculature and these lead to defects in adult behaviors such as flight, motility and fertility (Caygill & Johnston 2008 & Sokol et al., 2008). In the case of mammals, there are no detectable levels of *let-7* in embryonic stem cells and the *let-7* levels increase during embryogenesis and brain development (Schulman et al., 2005; Wulczyn et al., 2007).

Furthermore, *let-7* miRNA has been suggested to directly target cell cycle genes (Chen et al., 2010). Microarray analyses performed by Johnson and colleagues revealed many genes regulating cell cycle and cell proliferation that are responsive to alteration of *let-7* levels, including cyclin A2, CDC34, Aurora A and B kinases (STK6 and STK12), E2F5, and CDK8. Furthermore, *let-7* also inhibits several components of DNA replication machinery, transcription factors and checkpoint regulators (Johnson et al., 2007; Bueno and Malumbres 2011). Two recent publications report CDC25A as a likely target of *let-7*b in human cell culture, although it has remained unclear whether this interaction is physiologically relevant (Johnson et al., 2007, Huang et al., 2007).

In all these interactions, *let-7* acts as a negative regulator of its targets thus pointing towards the primary role of *let-7* as a potent inhibitor of cell proliferation and inducor of cell differentiation

3.11. Interfacing of the *let-7* miRNA in the heterochronic pathway to the cell cycle machinery

It is well established that proper development of the worm requires activation of specific genes in the heterochronic pathway at appropriate time to regulate the timing of the cell division and differentiation (Ambros and Horvitz 1984). However, it is unclear how these heterochronic gene products actually interface with the cell cycle machinery to regulate timing of the cell division and differentiation.

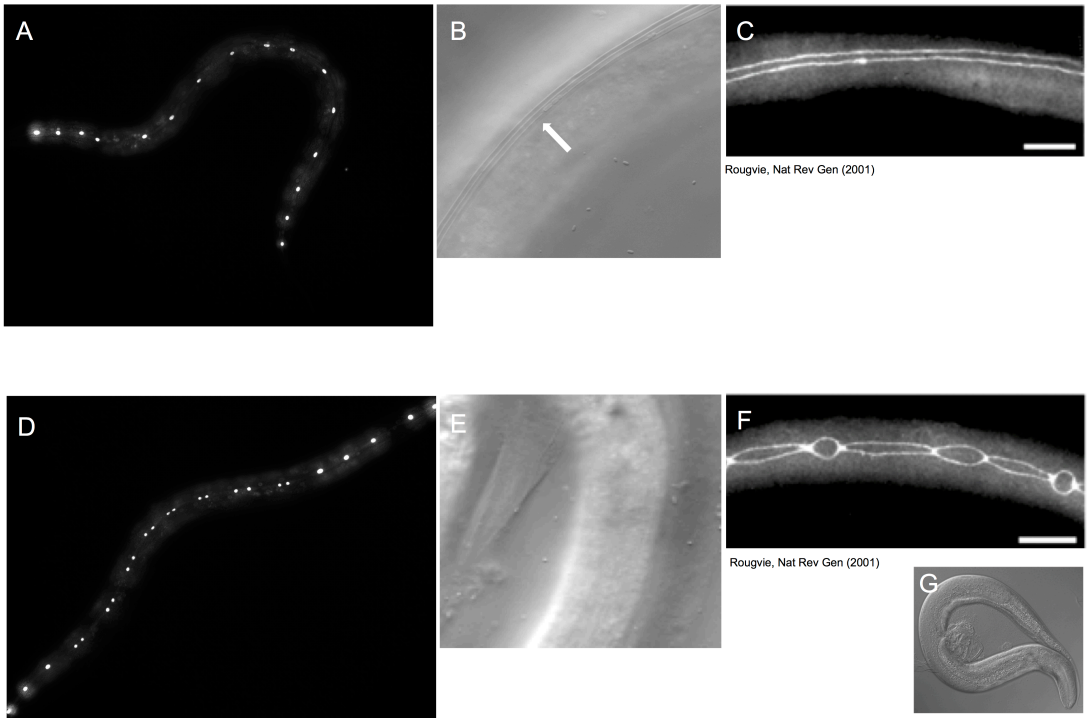


Figure 4. Seam cell phenotype in *let-7* mutant worms
A. In a wild-type worm there are 16 seam cells in adults. B. During larval-to-adult transition seam cells differentiate forming alae C. and fuse forming a syncytium.
D. In *let-7* mutant worms seam cells continue to proliferate. E. There is lack of differentiation i.e. no alae F. No fusion of seam cells is observed in *let-7* mutant worms. G. *let-7* mutant worms die by bursting through their vulva. Images in C and F taken from Rougvie 2001.

The effect of *let-7* mutation can be observed in a subset of hypodermal cells known as the seam cells, which show perturbed temporal cell fate patterns. Seam cells divide asymmetrically during each larval stage (Sulston and Horvitz 1977). However, during the (L/A) transition, when *let-7* levels are high, seam cells exit the cell cycle (Reinhart et al., 2000); they terminally differentiate fusing into a syncytium and secret an adult cuticular structure known as alae (Singh and Sulston 1978). Conversely, in the *let-7* mutant animals the seam cell not only continue their division even in the adult worm due to failure in exiting the cell cycle during the (L/A) transition but also continue to produce larval cuticle and fail to differentiate (Reinhart et al., 2000). Moreover, *let-7* function is essential for viability of these worms, since the *let-7(mn112)* null and the temperature-sensitive *let-7(n2853ts)* mutant strains at the restrictive temperature of 25°C, die by bursting through their vulva during the (L/A) transition (Reinhart et al., 2000). Interestingly this lethality can be suppressed by depletion of individual *let-7* targets using RNA interference (Grosshans et al., 2005).

Although we know that *let-7* directs cell differentiation during (L/A) transition in *C. elegans* through the regulation of one of its major down stream targets, *lin-41* (Slack et al., 2000), there are many open questions that still need to be answered and the exact mechanism that links *let-7* to the cell cycle machinery still eludes researchers. Thus investigating how *let-7* interfaces with the cell cycle machinery would provide us with a better understanding about how *let-7* mediates regulation of cell proliferation and differentiation and also help us assess the role played by its targets.

3.12. *lin-41 (lineage variant 41)* is TRIM-NHL protein

lin-41 is a heterochronic gene that was discovered in *C. elegans* and it is a downstream target of *let-7* miRNA. (Slack et al., 2000). The interaction between *let-7* miRNA and *lin-41* was extensively studied as a model of miRNA mediated down regulation and is highly conserved across species as diverse as nematodes to humans (Schulman et al. 2005; Lin et al. 2007). *lin-41* belongs to the TRIM-NHL family of proteins that are defined by the presence of N terminal tripartite motif [TRIM] consisting of a RING finger domain, B-Box and coiled-coil motif coupled to the NHL repeats (named after *N*CL-1, *H*T2A and *L*IN-41) near the C terminal end of the protein (Slack and Ruvkun 1998).

3.13. Functional insight into the TRIM-NHL proteins

The general feature in many TRIM-NHL proteins is its well-conserved domain architecture. With few exceptions, these proteins consist of five domains. Furthermore, the intricate domain architecture of TRIM-NHL proteins suggests that these proteins may involve several different modes of functioning mechanistically, where each domain may play a critical role in regulating development (Wulczyn et al., 2011). For instance in human TRIM32 protein two distinct developmental disorder arise due to mutation in two different domains of the protein. Mutation in the B-Box results in a Bardet-Biedl Syndrome (Chiang et al., 2006) whereas the mutation in the NHL domain of the same protein leads to Limb Girdle Muscular Dystrophy (Saccone et al., 2008).

Study of multiple TRIM-NHL proteins have unveiled possible roles played by each domain. Starting from the N-terminus, the first domain usually found in TRIM-NHL proteins is the RING, which is a zinc finger type domain that was found in the human RING1 (Freemont et al., 1993; Saurin et al., 1996). The RING domain possesses an intrinsic E3 ubiquitin ligase activity and many RING finger domain containing proteins play critical role in ubiquitination pathway (Deshaies et al., 2009). For example proteins such as Trim32 (Kudryashova et al., 2005) and TRIM2 (Balastik et al., 2008) show ubiquitin ligase activity. Furthermore, study of TRIM32 have revealed Ring domain in the Trim32 acts as an E3 ubiquitin ligase and promotes degradation of several targets, including actin (Kudryashova et al., 2005), Abl interactor 2 (Kano et al., 2008), X-linked inhibitor of apoptosis (XIAP) (Ryu et al., 2011), p73 transcription factor (Gonzalez et al., 2013), and thin filaments and Z-bands during fasting (Cohen et al., 2012). However, there are exceptions which entirely lack the RING domain. One such example is Dappled, which is a putative ortholog of *lin-41* in Drosophila. Additional TRIM-NHL proteins that lack the RING domain are *C. elegans* NCL-1 and Drosophila Brat, are observed as well.

In TRIM-NHL proteins the N terminal RING domain is usually followed by one or two B Box-type zinc finger domains that are of type 1 or type 2. B-Box domains are also implicated in ubiquitination (Massiah et al 2006).
The final domain in the TRIM consists of a coiled-coil motif. Coiled-coil is a highly versatile domain, which is also found in many different proteins (Lupas et al., 1996; Grigoryan et al., 2008). The alpha helices are coiled together like the strands of a rope to form dimers or trimers (Liu et al., 2006). Coiled-coil motif has been suggested to play important role in protein-protein interaction. An example of coiled-coil mediated protein-protein interaction was Trim32 protein, which was shown to physically interact with the head and neck region of the myosin heavy chain (Saccone et al., 2008). Furthermore, a study

carried out by Loer et al (2008) suggested that Dappled/Wech coiled coil and B-Box domains are involved in its binding to the ILK and Tensin.
A less commonly found domain in the TRIM-NHL proteins is the filamin-like domain, also known as Ig-filamin repeat. The Filamin domain is situated between the TRIM motif and the NHL repeats. The Function of the filamin like domain in TRIM-NHL proteins is unclear. However, they are thought to be involved in RNA repression and protein-protein interaction (Zhou et al., 2010).
The C terminal end of the TRIM-NHL proteins contains a domain consisting of multiple NHL repeats. Several studies have revealed that mutations altering the LIN-41 protein function map to the NHL domain, suggesting that the NHL repeats play a critical role in proper functioning of the TRIM-NHL proteins (Slack et al., 2000; Spike et al., 2014; Tocchini et al., 2014). The crystal structure of the isolated NHL from Brat has been solved and revealed that the NHL repeats forms a six-bladed propeller like structure very similar to the WD-40 B propeller (Edwards et al., 2003). Functionally the NHL repeats have been implicated in mediating RNA repression by directly binding to the RNA molecule as well as suggested to be involved in protein-protein interaction (El-Husseini and Vincent 1999; Saccone et al., 2008; Loedige et al., 2014). One example of a missense mutation in the Trim32 NHL domain which was implicated in a human disease known as the Limb-girdlemuscular dystrophy that affect both the ability of the protein to self-interact and to bind E2 (Saccone et al., 2008). Furthermore, Loedige and colleagues showed that the Drosophila protein brain tumor also known as Brat directly interacts with the hunchback mRNA mediating translational repression by Brat (Loedige et al., 2014).

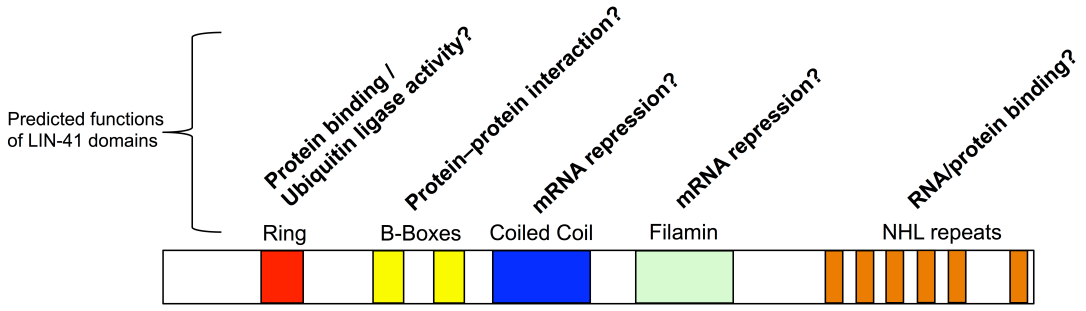


Figure 5. LIN-41 domain architecture
LIN-41 protein consists of RING domain near the N terminal followed by two B-Boxes, coiled-coil, Filamin and six NHL repeats near the C terminal end. Predicted functions of the individual domains are highlighted suggesting that LIN-41 may exert its function in diverse modes

3.14. LIN-41 is involved in developmental regulation of multiple tissues

Slack and colleagues observed LIN-41 protein expression in muscle cells, neurons, somatic gonad and lateral hypodermal seam cells (Slack et al., 2000). The pleiotropic nature of *lin-41*'s function can be assessed using gain- or loss-of-function mutants that affect development in variety of tissues, which display phenotypes ranging from gut defects, cell over-proliferation defect in the hypodermis to a more severe vulval defect phenotypes that result in death of the worms by bursting through the vulva (Slack et al., 2000). In addition to somatic defects *lin-41*'s role in germline as been well studied. *lin-41* plays a significant role in the germline development and is essential in the formation of oocytes (Tocchini et al., 2014 and Spike et al., 2014) and loss of function of *lin-41* causes sterility. All these studies suggest a broad role played by LIN-41 during the worm development.

In the soma, down-regulation of *lin-41 by let-7* is critical for larval-to-adult transition and has been well studied in the hypodermis of *C. elegans*. Genetic data supported *let-7* negatively regulating *lin-41* in a post-transcriptional manner by binding to the 3'untranslated region in the *lin-41* mRNA (Slack et al., 2000; Vella et al., 2004). This binding resulted in translational inhibition (Ding and Grosshans 2009) as well as mRNA degradation (Bagga et al., 2005). *lin-41*'s function is well characterized in seam cells, where it was shown to regulate cell proliferation and differentiation. It was observed that loss of LIN-41 resulted in precocious execution of the larval-to-adult transition, where the seam cells exit cell cycle early during the development and differentiate in the L4 stage. In contrast, similar to the phenotype observed in

let-7 loss-of-function worms, LIN41 overexpression resulted in a retarded larval-to-adult transition, resulting in some seam cells undergoing an additional round of division, not only increasing the seam cell numbers in the adult stage but also preventing them from fusing and differentiating (Reinhart et al., 2000; Slack et al., 2000). Knock down of *lin-41* resulted not only in the suppression of the bursting but also restored the temporal cell fate perturbation in the seam cells caused due to *let-7* loss-of-function. Current data suggest that *let-7* miRNA primarily acts through *lin-41*, since the aberrant seam cell divisions are completely suppressed by *lin-41* RNAi, but not another *let-7* target *hbl-1* (Vadla et al., 2012). Moreover, *lin-41's* orthologs were shown to play a crucial role in vertebrate development by studying loss-of-function mutants in zebrafish and mice. In both organisms, *lin-41* expression correlated with proliferative progenitor tissue. Loss of *lin-41* function results in embryonic lethality in Zebra fish (Lin et al., 2007) as well as mouse embryos (Chen et al., 2012).

3.15. LIN-41 functions as a post-transcriptional regulator of gene expression

Interestingly it was proposed that *lin-41* itself was a post-transcriptional regulator, acting as a translational repressor of a down stream transcription factor *lin-29* (Slack et al., 2000). Supporting this evidence was the fact that *lin-29* mRNA accumulates two larval stages before LIN-29 protein becomes detectable (Rougvie and Ambros 1995, Bettinger et al., 1996), and that *lin-41(lf)* mutations cause precocious LIN-29 accumulation in seam cells (Slack et al., 2000). However, the mechanism of this regulation has remained elusive. More recent studies provided a mechanistic insight into how LIN-41 may regulate its targets. In the same year, two studies involving *in vitro* cell culture based on HEK 293 and mES cells observed that modulating LIN-41's amount not only changed the mRNA but also affected the protein levels (Chang et al., 2012; Loedige et al., 2012). Furthermore, it was revealed that the 3'UTR was sufficient to mediate the LIN-41 based translational repression of a luciferase reporter (Chang et al., 2012; Loedige et al., 2012). Loedige and colleagues further observed that this repression was attributed to the coiled-coil and filamin domains of the protein, while RNA binding was exclusively dependent on the NHL repeats (Loedige et al., 2012). However, the mode of *lin-41* recruitment on mRNA remains to be solved, since no consensus sequence or binding motif that would explain the mRNA interaction with *lin-41* has been found. In addition although LIN-41 is suggested to bind proteins, many of these interactions were indirect and mediated via mRNA (Loedige et al., 2012).

Another proposed mode of how LIN-41 may function was based on the E3 ubiquitin ligase activity due to the presence of a TRIM domain (Slack et al., 2000). In a study carried out by Rybak and colleagues, mouse LIN-41 not only mediated Argonaute protein Ago2 ubiquitylation in the stem cells, which resulted in the degradation of Ago2 (Rybak et al., 2009) but also showed that LIN-41 could ubiquitylate itself (Rybak et al., 2009; Loedige et al., 2012). However, the physiological relevance of the LIN-41 based ubiquitylation remains elusive. Furthermore, at least in *C. elegans* an observation that argues against LIN-41 mediated ubiquitylation as its major molecular activity is that, none of the LIN-41 loss-of-function mutants map to the RING domain. Interestingly many studies carried out using EMS mutagenesis screen that resulted in measurably phenotype were mapped to the NHL domain (Slack et al., 2000; Tocchini et al., 2014; Spike et al., 2014). Another indication that argues against ubiquitylation as a major function of LIN-41 is the lack the RING domain in Drosophila LIN-41 homologue dappled/wech (O'Farrell et al., 2008).

3.16. Evidence of *lin-41* as a promoter of cell proliferation and inhibitor of differentiation by targeting cell cycle regulators

Several TRIM-NHL proteins have been implicated in regulation of self-renewal and differentiation. TRIM-NHL proteins such as BRAT, NHL-2 MEI-P26 are generally known to function as inhibitors of cell proliferation and promote differentiation (Frank et al., 2002; Hammel et al., 2009; Neumuller et al., 2008), LIN-41 on other hand has been found as a cell cycle promoting factor. Recent studies have provided further insight about how LIN-41 regulates these two antagonistic processes by revealing its downstream targets, many of which are factors that are directly involved in cell cycle regulation. For instance in the mouse embryonic stem cells, TRIM71, an ortholog of LIN-41 (Kanamoto et al., 2006), was found to repress cyclin-dependent kinase inhibitor CDKN1A thereby promoting G1-to-S phase transition (Chang et al., 2012).

Additional evidence of Lin-41's involvement in regulating cell cycle came from study carried out by Loedige and colleagues in mouse ES cells, where they demonstrated that TRIM71 targets transcription factors and cell cycle regulators RBL1 and RBL2 on the mRNA level thus down-regulating them (Loedige et al., 2012).

A recent study carried out in human induced pluripotent stem cells revealed that knock-down of *let-7* or over expression of *lin-41* resulted in an increase in the efficiency of iPSC reprogramming. Additionally it was suggested that LIN-41 directly represses the transcription factor EGR1 (Worringer et al., 2014).

Thus the overall picture points towards *lin-41* and its orthologs playing a critical role in regulating cell proliferation and differentiation.

4. Project I: "Cell cycle regulation by *let-7* microRNA and coordination with cellular differentiation"

4.1. Background and Aims

miRNAs play a key role in the regulation of multiple biological processes. Since their discovery in 1993 (Lee et al., 1993), numerous studies have implicated altered miRNA expression as well as loss of regulation of genes controlled by miRNA in many disorders, such as cancer, cardiovascular and neurodegenerative diseases (Ardekani and Naeini 2010).

miRNAs can mediate gene expression regulation by affecting multiple targets (Brennecke et al., 2005; Giraldez et al., 2006; Hashimoto et al., 2013). Thus miRNA target recognition is critical to understanding the function of miRNA and its regulation. Although there is general consensus about the mechanism behind miRNA targeting, which involves the interaction between the seed region of a miRNA, which is a conserved 6-8 nucleotide long sequence, mostly situated at positions 2-7 from the miRNA 5´-end, and target 3'UTR region (Lewis et al., 2005; Huges et al., 2011; Grimson et al., 2007), accurately predicting miRNA target still remains a challenge (Rajewsky 2006). Several algorithms have been developed to identify miRNA targets. However, effective prediction of the interaction between miRNA-mRNA has proven to be difficult due to limited knowledge of rules governing these processes. Thus experimental validation of miRNA-target interactions is the only certain way of validating the physiologically relevant targets (Bartel, 2009; Thomson et al. 2011; Witkos et al. 2011).

The aim of this project is to assess the direct *let-7* targets involved in regulation of cell cycle pathway that are physiologically relevant and in the process attempt to gain insight into the role played by *let-7* miRNA in coordinating cell cycle regulation and differentiation process in the seam cells.

A former PhD student, Almuth Mullner initiated the project by screening >40 genes that are predicted to function as cell cycle regulators. By performing RNAi against the candidate genes, Almuth not only tested for suppression of the bursting phenotype in the loss-of-function *let-7* mutant worms but also screened for the reversal of hypodermal phenotypes i.e. seam cell over-proliferation and lack of differentiation. The results thus obtained from the initial screen were used as a base for the detailed analysis that I performed. I repeated a part of the initial screen for our candidate genes in order to confirm Almuth's observation. After validating the preliminary results, I went on to

create reporter strains that enabled us to assess direct *let-7* mediated regulation of candidate genes from the screen.

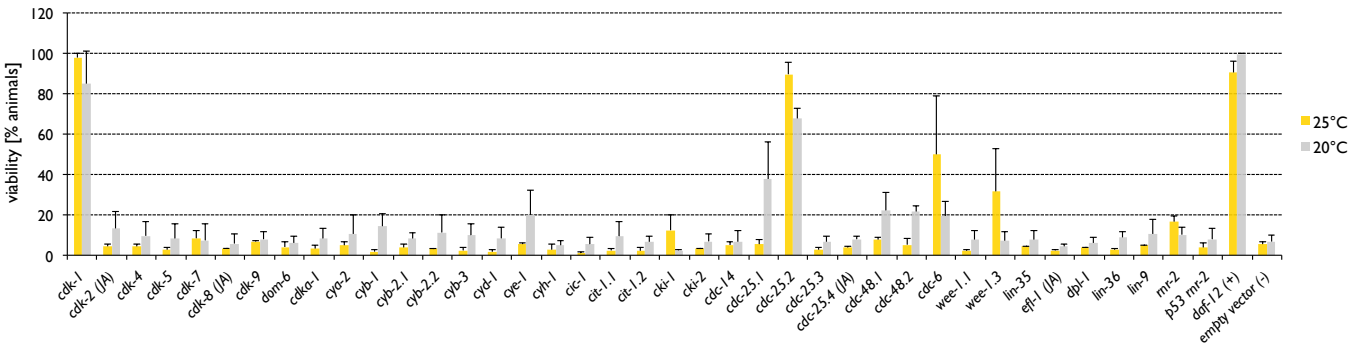
4.2. Results

4.2.1. RNAi screen against cell cycle regulators to identify suppressors of *let-7(n2853ts)* bursting

let-7 miRNA is a key heterochronic gene involved in larval-to-adult (L/A) transition in *C. elegans* (Reinhart et al., 2000; Grosshans et al., 2005). The *let-7(n2853ts)* allele contains a point mutation in the *let-7* seed sequence. At 15°C, *let-7(n2853ts)* animals display the retarded seam cell phenotype described above, and at 25°C they die as young adults by bursting through the vulva, as do *let-7(mn112)* null mutants at any temperature. This lethality can be suppressed to varying extents by depletion of individual targets by RNAi, suggesting that this drastic phenotype results from the misexpression of several *let-7* target genes (Grosshans et al., 2005). This fact provided a convenient approach for identification of potential *let-7* target genes. Based on the annotations for known or predicted cell cycle regulators in the *C. elegans* Wormbase database, >40 genes were tested to see if their depletion by RNAi suppressed the *let-7(n2853ts)* bursting phenotype at non permissive temperature of 25°C. The suppressor screen resulted in six candidate genes *cdk-1, cdc-25.2, mcm-7, cdt-1, cdc-6* and *wee-1.3*, which suppressed the *let-7(n2853ts)* lethality caused by vulval bursting to varying degrees (Fig. 6A). Out of these six genes, we observed *cdk-1*, *cdc-25.2, mcm-7* and *cdt-1* resulting in worms showing rescue of bursting phenotype of 97 %, 89 %, 97% and 96% respectively (n>100). In the case of *cdc-6* and *wee-1.3* we observed the bursting phenotype rescue of 50% and 31% respectively. In addition we also tested these genes for the lethality suppression in the *let-7(mn112)* null mutant. While RNAi against *cdk-1*, *cdc-25.2, mcm-7* and *cdt-1* resulted in greater than 95% of the worms rescued, *cdc-6* and *wee-1.3* resulted in only 11.3% and 4.8% rescued worms respectively (Fig. 6B). These results suggested that *cdc-25.2*, *cdk-1, mcm-7* and *cdt-1* are particularly interesting.

However, upon closer inspection of these worms, we found out that, when grown under *cdk-1* or *cdc-25.2* RNAi conditions, about half of the surviving worms were vulvaless (Fig. 7). This contrasts with RNAi against the established *let-7* targets- *lin-41* or *hbl-1* (Slack et al., 2000; Abrahante et al., 2003), which suppressed the *let-7* bursting phenotype, however, did not display the vulvaless phenotype. This observation led us to wonder if the suppression of the *let-7* bursting phenotype under *cdk-1* and *cdc-25.2* RNAi condition was non-specific.

A


viability [% animals]
120
100
80
60
40
20
0
25°C
20°C
cdk-1
cdk-2 (JA)
cdk-4
cdk-5
cdk-7
cdk-8 (JA)
cdk-9
dom-6
cdka-1
cya-2
cyb-1
cyb-2.1
cyb-2.2
cyb-3
cyd-1
cye-1
cyh-1
cic-1
cit-1.1
cit-1.2
cki-1
cki-2
cdc-14
cdc-25.1
cdc-25.2
cdc-25.3
cdc-25.4 (JA)
cdc-48.1
cdc-48.2
cdc-6
wee-1.1
wee-1.3
lin-35
efl-1 (JA)
dpl-1
lin-36
lin-9
mr-2
p53 mr-2
ddf-12 (+)
empty vector (-)

B

	let-7(n2853)		*let-7(mn112)*
RNAi	**25°C a) [%]**	**20°C a) [%]**	**25°C a) [%]**
empty vector (-)	**5.3 (1.1)**	**6.8 (3.3)**	**3.2 (1.4)**
daf-12 (+)	**90.5 (5.5)**	**99.7 (0.4)**	**59.8 (16.9)**
cdk-1	**97.9 (2.3)**	**85.0 (16.1)**	**95.7 (1.4)**
cdc-25.2	**89.7 (5.7)**	**67.7 (5.2)**	**98.3 (1.9)**
mcm-7	**97.2 (n.d.)**	**n.d.**	**99.0 (n.d.)**
cdt-1	**96.2 (n.d.)**	**n.d.**	**99.2 (n.d.)**
cdc-6	**50.0 (28.6)**	**19.3 (7.4)**	**11.2 (10.4)**
wee-1.3	**31.5 (21.1)**	**7.0 (4.5)**	**4.8 (3.9)**

C

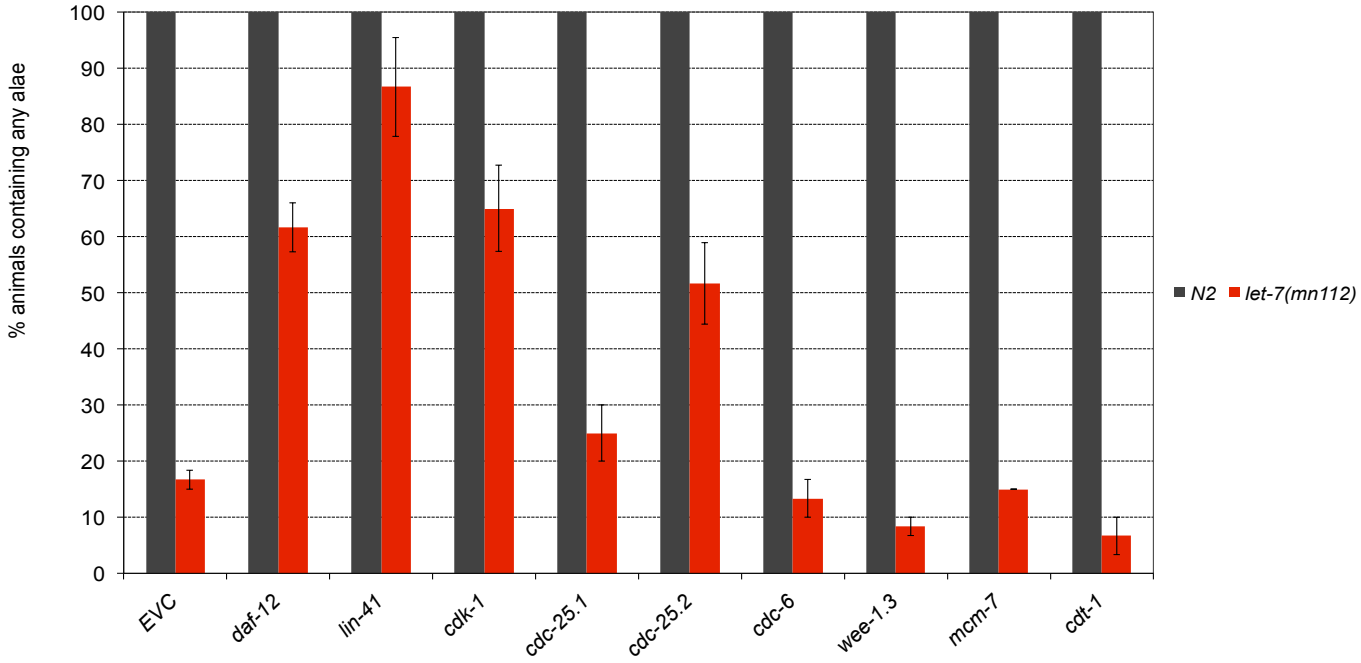


Figure 6. An RNAi screen to assess cell cycle genes for the suppressors of *let-7* mutant's bursting phenotype.

A. In order to assess the potential *let-7* targets, the suppression of the bursting phenotype in *let-7(n2853)* mutant worms was assessed by knock-down of genes involved in regulation of cell cycle. RNAi performed by feeding bacteria. Synchronized larval stage 1 worms were put onto RNAi plates. Suppression of the bursting phenotype was tested at two temperatures 25°C and 20°C. The worms were analysed during adult stage. RNAi depletion of six genes *cdk-1, cdc-25.2, mcm-7, cdt-1, cdc-6*, *wee-1.3,* resulted in suppression of the bursting in *let-7* mutant worms. *daf-12* an established *let-7* target served as a control.

B. % of worms showing suppression of bursting by performing RNAi against six cell cycle genes in *let-7(n2853)* mutant worms and *let-7(mn112)* null animals. RNAi against *cdk-1, cdc-25.2, cdt-1* and *mcm-7* resulted in strong bursting phenotype suppression. a) Indicate mean of three independent biological experiments with number of animals >90 per condition and replicate (Standard deviation).

C. Hypodermal phenotype observed in the *let-7 (mn112)* worms was investigated by assessing alae formation i.e. differentiation in the seam cells**.** Only *cdk-1* and *cdc-25.2* resulted in restoration of alae upon their knock-down in *let-7 (mn112)* background*. daf-12 and lin-41* served as positive controls. Error bars indicate SEM from three independent biological experiments. Number of animals scored per replicate= 20.

However, when we looked at the seam cells, which display over proliferation defects as well as lack of differentiation in the *let-7* mutant worms, we observed that RNAi mediated depletion of *cdk-1* and *cdc-25.2,* but not *mcm-7* or *cdt-1,* resulted in alae restoration in *let-7(n2853ts)* as well as *let-7(mn112)* animals, confirming the specificity of this genetic interaction. We observed that only 9% (n=32) of *let-7(mn112)* animals on mock RNAi displayed any alae, where as 51% (n=47) of animals on *cdk-1* RNAi and 41% (n=27) of animals on *cdc-25.2* RNAi did. Similar to the *lin-41* RNAi positive control, knockdown of *cdk-1* and *cdc-25.2* virtually always resulted in partial, rather than complete alae. Despite the vulvaless phenotype observed under *cdk-1* and *cdc-25.2* RNAi conditions (Fig. 7), the restoration of alae in both the *let-7* mutants (Fig. 6C) suggested the possibility that these genes may be *let-7* targets. To look at this more closely we also looked at the seam cell number and found that both *cdk-1* (Mean= 15.5 seam cells, SD= 1.235 n=20) and *cdc-25.2* (Mean= 14.7 seam cells, SD= 1.341; n=20) RNAi resulted in reduction of additional seam cell divisions versus *EV* (Mean= 20.05 seam cells, SD= 2.305; n=20). Values are statistically significant based on TTest *cdk-1* (p value= 2.74218E-12) & *cdc.25.2* (p value= 1.28165E-14) TTest- 1 tailed, unpaired (Almuth Müllner, personal communication).

Additionally, we also tested these genes for their specific role in hypodermal differentiation using a *gfp* reporter driven by the *col-19* promoter in *let-7(n2853)* animals. COL-19 is an adult specific collagen whose expression depends on *let-7* activity and it is therefore absent in *let-7* mutant worms (Thein et al., 2003). Both *cdk-1* and *cdc-25.2* RNAi resulted in up-regulation of *col-19::gfp* in the hypodermis, which further supported the specificity of this genetic interaction. Interestingly upon closer inspection of the worms, *cdk-1* and *cdc-25.2* knockdown resulted in *col-19::gfp* expression in hyp7 but not the seam cells, which was surprising considering that hyp7 is post-mitotic. Moreover, RNAi against *let-7's* direct target- *lin-41* resulted in up-regulation of *col-19* not only in the hyp7 cells but also in the seam cells (Fig. 8).

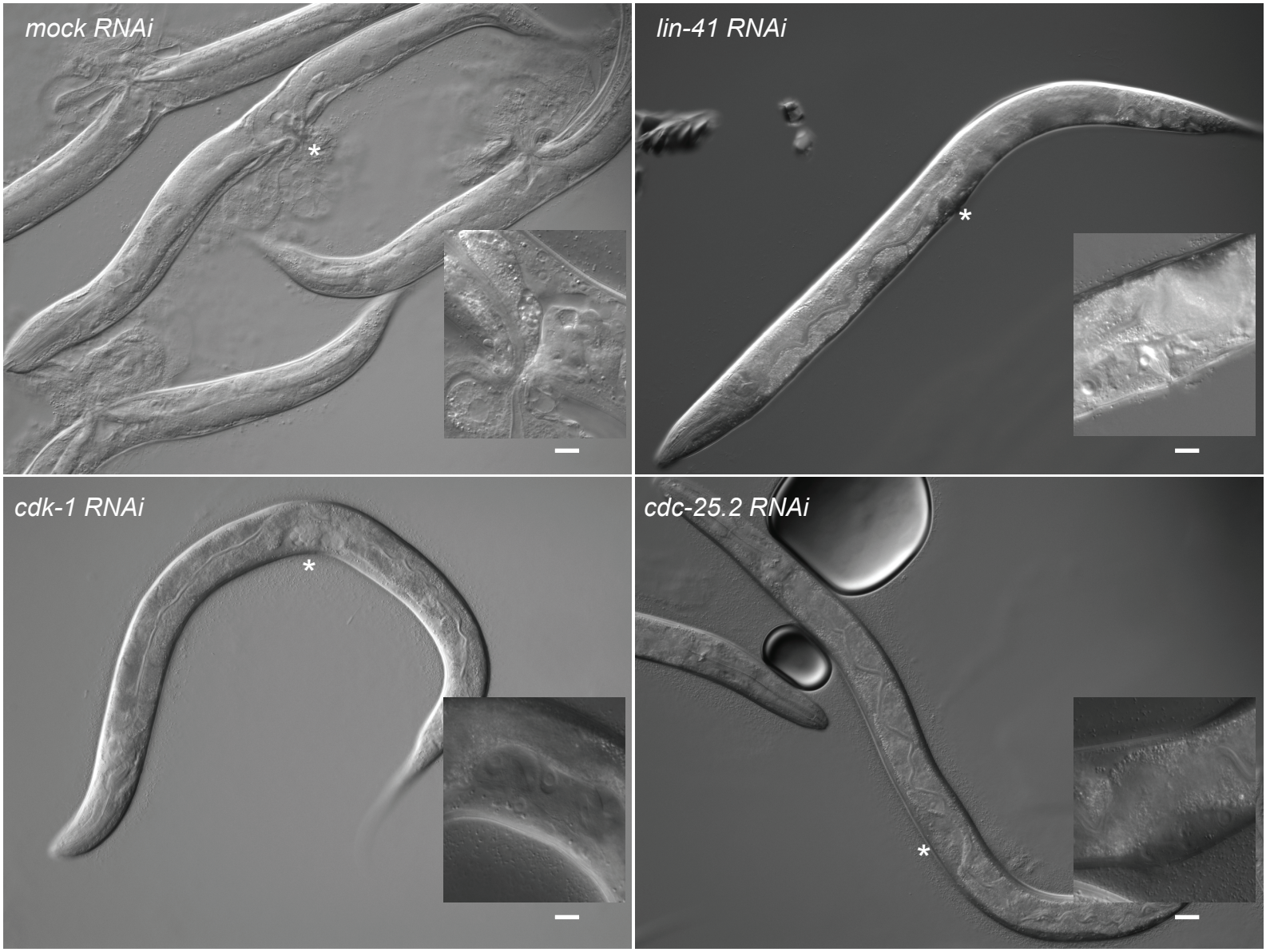


Figure 7. *cdk-1* or *cdc-25.2* RNAi results in partly penetrant vulvaless phenotype.
Suppression of the bursting phenotype in *let-7(n2853ts)* worms observed under *cdk-1* and *cdc-25.2* RNAi conditions may be partly due to the vulvaless phenotype observed in these worms.
Worms grown under *mock* RNAi burst through their vulva during adult stage.
lin-41 served as a control, since it is an established target of *let-7.* Knock down of *lin-41,* results in suppression of the bursting in *let-7(n2853ts)* worms. However, *lin-41* RNAi does not result in the vulvaless phenotype observed under *cdk-1* and *cdc-25.2* RNAi conditions. Vulvae are marked with asterisks. Scale bar indicates 20 um.

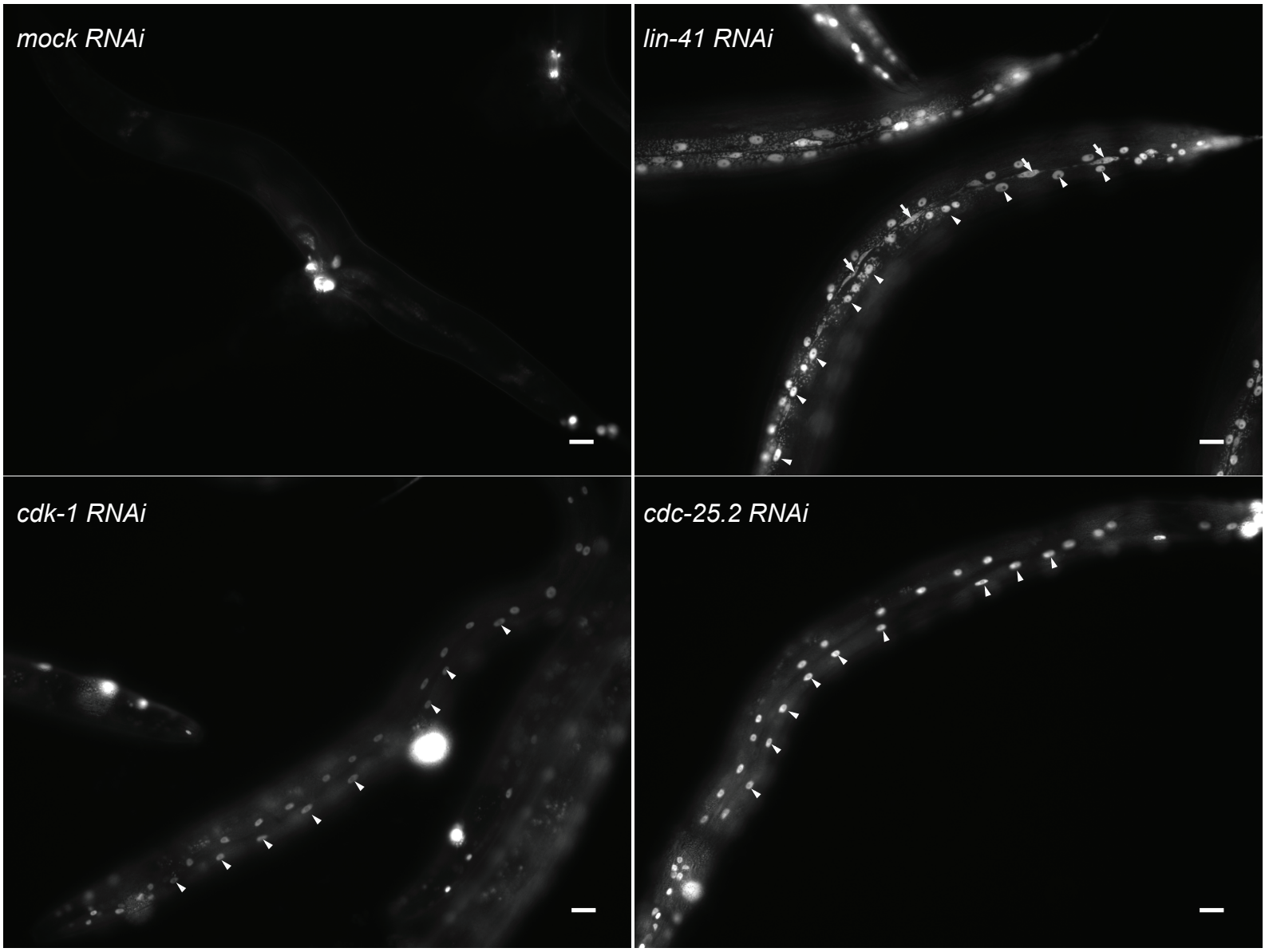


Figure 8. RNAi against *cdk-1* or *cdc-25.2* results in up-regulation of a *col-19::gfp* reporter in hypodermis

cdk-1 and *cdc-25.2* knockdown resulted in up regulation of *col-19::gfp* expression only in hyp7 (arrow heads) but not the seam cells (arrow) in *let-7(n2853ts)* worms. *mock* RNAi results in no up-regulation of the reporter in the *let-7* mutant background. RNAi against *lin-41,* which is an established target of *let-7* results in expression of *col-19::gfp* reporter in seam cells as well as the hyp7 cells. Scale bar indicates 20 um.

4.2.2. *let-7* does not bind to the 3'UTRs of *cdk-1* and *cdc-25.2*

Based on these results it seemed possible that *cdk-1* and *cdc-25.2* were direct targets of *let-7*. In order to assess this possibility we generated *cdk-1* and *cdc-25.2* 3' UTR reporters to assess their potential for regulation by *let-7*. As mentioned earlier, *let-7* functions by binding to the 3'UTR region of its target gene, thus resulting in mRNA degradation and/or translational repression. In order to assess *let-7* mediated regulation that encompasses both modes of regulation, and to assess *let-7* target regulation in a tissue-specific manner, we created a reporter system consisting of *gfp* fused to either *cdk-1* or *cdc-25.2* 3' UTR. The reporter was driven by the hypodermal specific *wrt-2* promoter (Aspöck et al., 1999) to assess their potential for regulation by *let-7* specifically in the seam as well as hyp7 cells. We termed these reporters as *pREP_cdk-1* and *pREP_cdc-25.2*.

When we analyzed *pREP_cdk1* and *pREP_cdc-25.2*, we found them both to be repressed in larval stage 4 animals in seam cells as well as the hyp7 syncytium relative to the unregulated *pREP_unc54* control reporter. For *pREP_cdk1* this repression was more pronounced in hyp7 than the seam, whereas the opposite was true for *pREP_cdc-25.2*. However, whereas the positive control pREP_*lin-41* was efficiently derepressed in the *let-7(n2853)* mutant background, this was not observed for *pREP_cdk-1* and *pREP_cdc25.2* in either tissue (Fig. 9). We conclude that although the 3' UTRs of these two mitotic genes might confer post-transcriptional repression at the larval stage four and beyond, when *let-7* is present, this seems unlikely to be a consequence of *let-7* function.

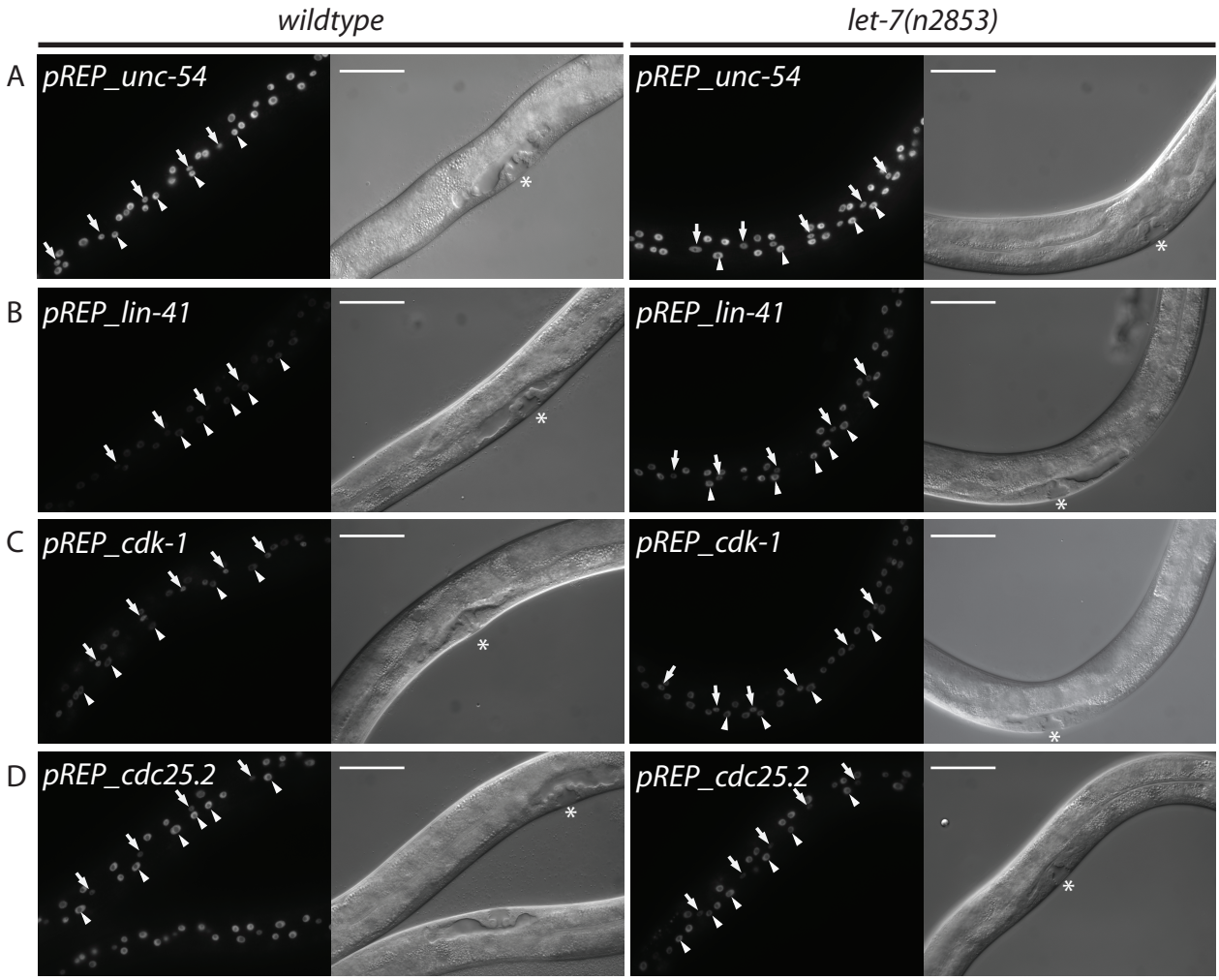


Figure 9. The 3'UTRs of *cdk-1* and *cdc-25.2* do not confer *let-7*-dependent regulation.

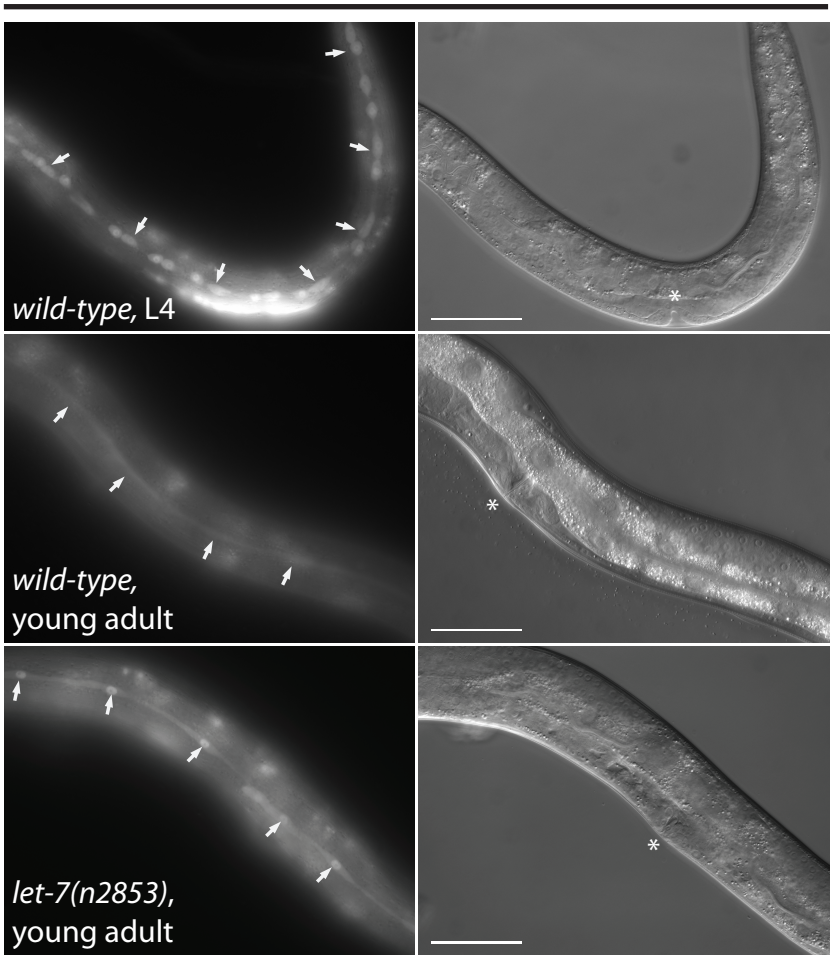
A. A hypodermis specific target reporter (*wrt-2* promoter) containing *gfp* fused to the unregulated *unc-54* 3'UTR (*pREP_unc-54*) is expressed both in wild-type and *let-7(n2853ts)* background at the late L4 stage. **B-D**. The reporter containing the *lin-41* 3'UTR (*pREP_lin-41*) is repressed in a *let-7* dependent manner (B), while repression of reporters carrying the cdk-1 (*pREP_cdk-1*, C) or *cdc-25.2* 3'UTR (*pREP_cdc-25.2,* D) in wild-type worms is less extensive and persists in the *let-7(n2853ts)* background. Vulvae are marked with asterisks. Scale bar indicates 50um .

4.2.3. *let-7* regulates CDK-1 expression in a LIN-29-dependent manner

We therefore wondered if *cdk-1* functioned further downstream of *let-7* in the heterochronic pathway. We utilized a previously published *cdk-1::gfp* single copy-integrated transgene, which drives expression of a functional fusion protein from the native *cdk-1* promoter (Shirayama et al. 2012), to examine the effect of *let-7* on CDK-1 accumulation. We observed that CDK-1/GFP was present in early L4-stage seam cells, but that its levels declined rapidly upon entry into adulthood. However, down-regulation was impaired in *let-7(n2853)* mutant animals where CDK-1/GFP was well visible in the seam cell cytoplasm and, prominently, nucleus (Fig. 10A).

To understand better why CDK-1/GFP protein levels responded so strongly to loss of *let-7* activity although *let-7* did not appear to repress it directly, we tested whether *cdk-1::gfp* expression was modulated by the downstream effector LIN-29 (Bettinger et al., 1996). Indeed, knock-down of *lin-29* by RNAi, resulted in elevated levels and redistribution of CDK-1/GFP, similar to the effect of *let-7(n2853).* A similar up-regulation was also observed for *mab-10* RNAi, a transcription co-factor that acts in concert with LIN-29 to promote differentiation of the hypodermis (Harris and Horvitz 2011). Thus, we conclude that *let-7* regulates *cdk-1* indirectly, in a manner that requires the LIN-29 transcription factor (Fig. 10B).

A


Pcdk-1::cdk-1-gfp::cdk-1 3'UTR
wild-type, L4
wild-type,
young adult
let-7(n2853),
young adult

B

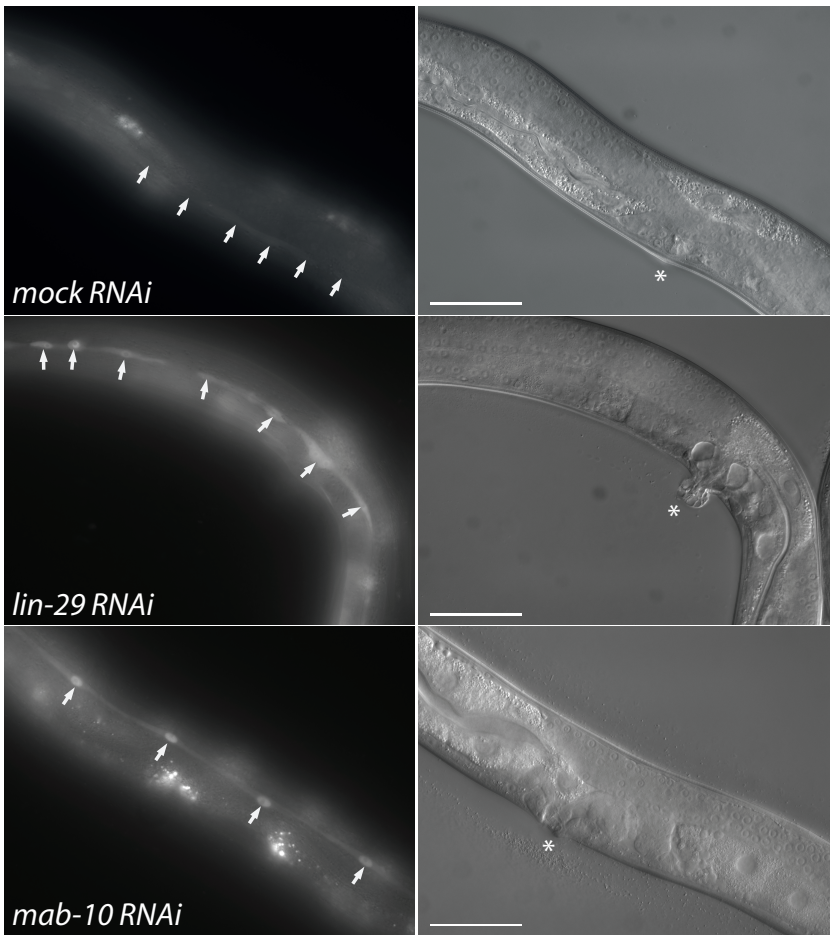

let-7 wild-type; Pcdk-1::cdk-1-gfp::cdk-1 3'UTR
mock RNAi
lin-29 RNAi
mab-10 RNAi

Figure 10. Repression of *cdk-1::gfp* depends on LIN-29 and MAB-10. **A.** Expression of *cdk-1::gfp* from the *cdk-1* promoter can be observed in seam cells (arrows) until the L4 stage. GFP levels decrease during L4 stage in wild-type background. *let-7(n2853ts)* mutant animals continue to express *cdk-1:gfp* in adult stage. **B.** Downregulation of *cdk-1::gfp* in wild type adult worms is lost upon RNAi-mediated knockdown of *lin-29* or *mab-10*. Vulvae are marked with asterisks. Scale bar represents 50 um.

5. Project II: "Functional Characterization of LIN-41 and its Targets"

5.1. Background and Aims

In the first project we demonstrated that at least for the two cell cycle genes *cdk-1* and *cdc-25.2,* we could not find any evidence supporting direct regulation by *let-7.* At the same time independent research in the lab showed that LIN-41 seems to be the key target of *let-7* function at least in the vulva (Ecsedi et al., 2015). Furthermore, work carried out by Vadla and colleagues demonstrated that *let-7* miRNA act through *lin-41*, since the aberrant seam cell divisions are completely suppressed by *lin-41* RNAi, but not another *let-7* target *hbl-1* (Vadla et al., 2012). Surprisingly little is know about how *lin-41* functions. Hence we decided to focus on *lin-41* in the second project.

Slack and colleagues showed that *let-7* miRNA targets *lin-41* 3'UTR, thus mediating its down-regulation (Slack et al., 2000). However, what happens downstream of *lin-41* is as yet unclear and although *lin-41* has been well studied in the context of miRNA based regulation, there is dearth of knowledge about how *lin-41* itself functions at molecular level in regulating the development of an animal. Only recently the picture has begun to change, where multiple studies have not only identified LIN-41 and its ortholog tripartite motif 71 (TRIM71) proteins as the regulators of stem and progenitor cell proliferation and differentiation, but also provided an insight into how *lin-41* may function in regulating these processes (Rybak et al., 2009; Chang et al., 2012; J Chen et al., 2012; Loedige et al., 2012; Worringer et al., 2014).

The preliminary evidence about how *lin-41* may function came from Slack et al (2000) paper, where they suggested that LIN-41 was involved in the negative regulation of a downstream protein LIN-29, which is a zinc finger transcription factor. LIN-29 protein is expressed starting from the larval stage 4 and continues to be expressed in the adult stage. LIN-29 is necessary to trigger multiple aspects of larval-to-adult transition, such as the expression of adult specific collagen gene *col-19*, cessation of molting cycle, as well as the seam cells exit from cell cycle and their terminal differentiation (Ambros and Horvitz 1984; Bettinger et al., 1996). In fact precocious defects in *lin-41* loss-of-function animals require functional LIN-29 protein (Slack et al., 2000). Interestingly *lin-29* mRNA is known to accumulate much earlier i.e. starting from larval stage 2, suggesting the possibility of *lin-29* regulation occurring at translational or protein level, thus restricting the time of the L4-to-adult transition only when LIN-29 protein is expressed (Bettinger et al., 1996; Slack et al., 2000). Surprisingly for more than a decade this question remained

unanswered, which prompted us to revisit it and try to understand not only the process through which LIN-41 regulates LIN-29, but also attempt to gain mechanistic insight into LIN-41's function by assessing factors that may bind to LIN-41 and are critical for LIN-41's function. In order to answer these questions I created a tagged version of LIN-41 protein, which enabled us to perform Co-IP experiment in order to assess both mRNA binding potential as well as protein binding partners of LIN-41. Together with Florian Aeschimann, I also generated *lin-29* reporter worm strain, which allowed us to gain critical insight into LIN-41 mediated regulation of LIN-29 protein.

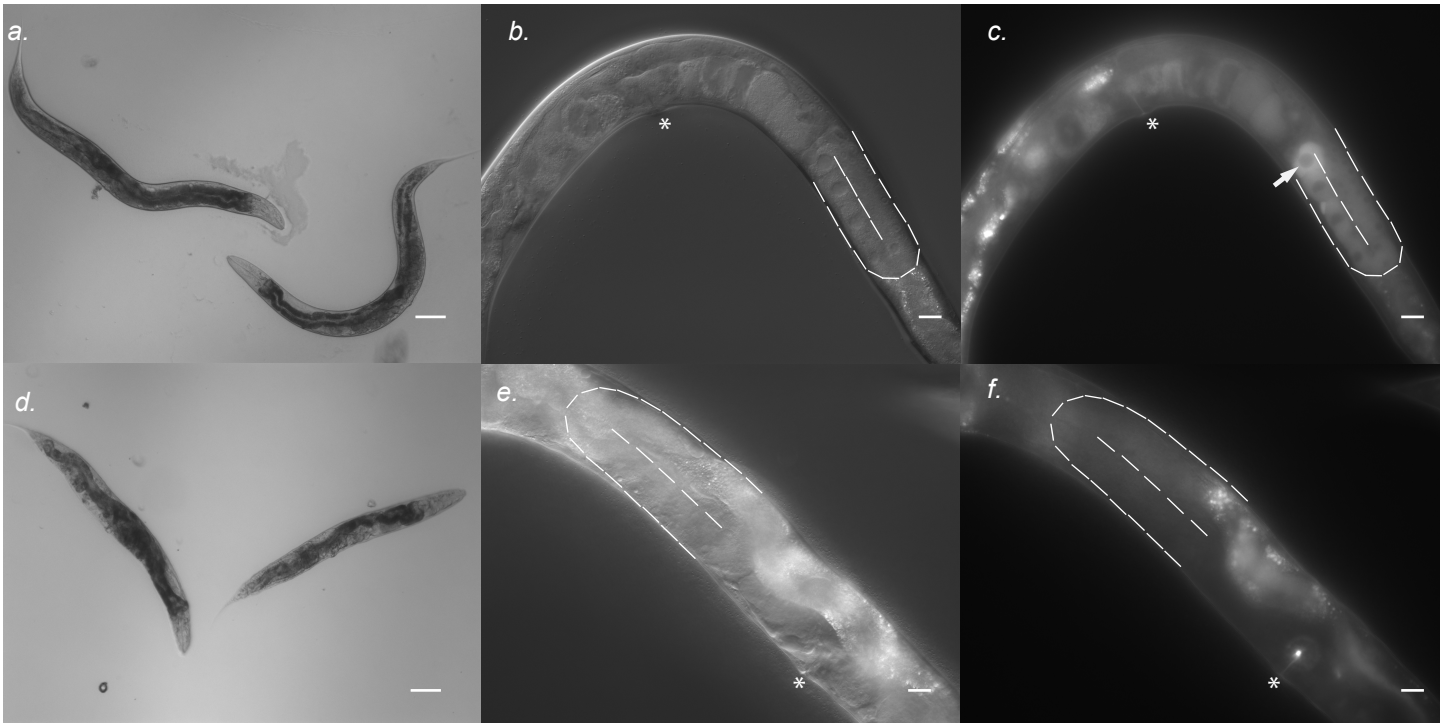
5.2. Results

5.2.1. Generating functional tagged version of LIN-41

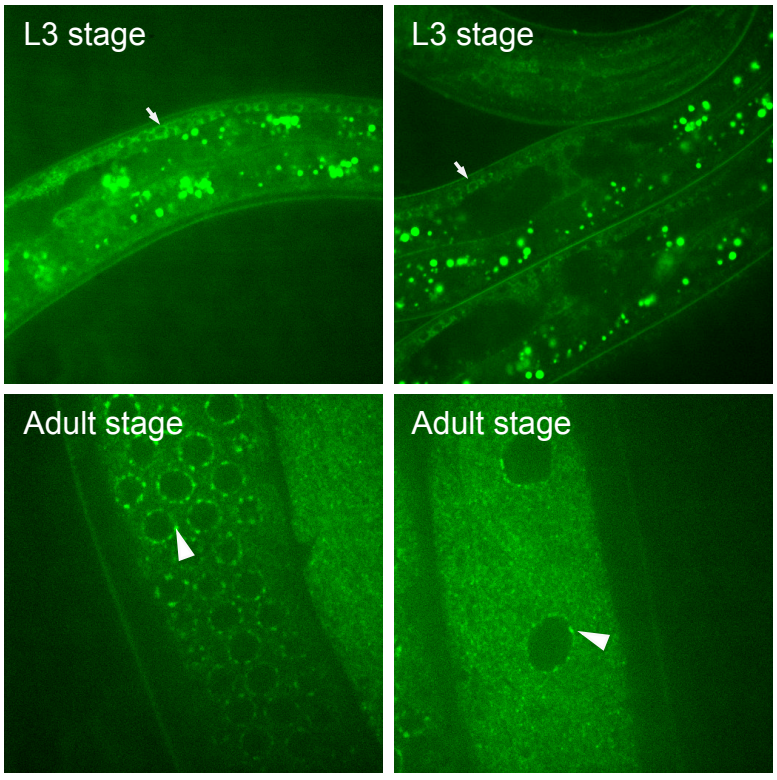
In order to investigate how *lin-41* functions, we attempted to create a tagged version of LIN-41 protein by generating worm lines expressing N-terminally 1xFLAG/GFP tagged LIN-41 as well as C-terminally 1xFLAG/GFP tagged LIN-41. Both versions of tagged *lin-41* were driven by *lin-41* promoter and included the *lin-41* 3'UTR. By using the MosSCI technique, we were able to integrate single copy of *lin-41* transgene, thereby avoiding the lethal phenotypes resulting from LIN-41 overexpression caused due to multicopy array, which was originally used in the Slack et al (2000) paper. In order to assess which of the tagged version was functional, we crossed the worms expressing transgenic *lin-41* into *lin-41(n2914)* null allele and observed that only N-terminally 1xFLAG/GFP tagged LIN-41 could fully rescue the *lin-41* null phenotypes of sterility as well and dumpiness.

Although Slack and colleagues described LIN-41 protein expression pattern, the data came from GFP tagged LIN-41 expressing from a multicopy array. Our single copy integrated GFP tagged version of LIN-41 enabled us to observe a functional LIN-41's *in vivo* expression pattern as well as the localization in individual cells under more physiological conditions. We observed weak expression of the tagged LIN-41 in several somatic tissues such as hypodermis, pharynx, neurons and muscle cells from early larval stage 2. From larval stage 4 onwards we saw down-regulation of LIN-41 in the soma (Fig. 11A). In the adult stage worms the somatic expression of LIN-41 ceased and we observed strong expression exclusively in the germline. Confocal microscopy analysis revealed LIN-41's granular distribution throughout the cytoplasm. Furthermore, we observed strong perinuclear localization of LIN-41 (Fig. 11B).

A


a.
b.
c.
d.
e.
f.
mock RNAi
gfp RNAi

B


L3 stage
L3 stage
Adult stage
Adult stage

C

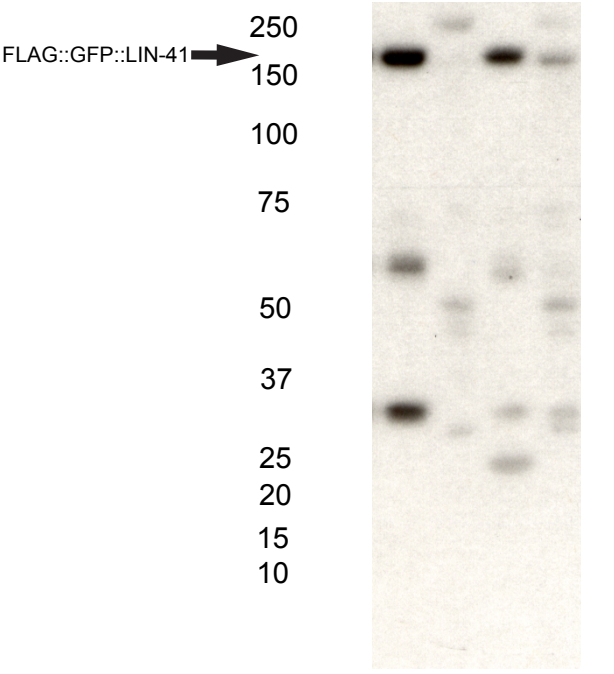

FLAG peptide elution
Unbound
Beads fraction
Input
FLAG::GFP::LIN-41
250
150
100
75
50
37
25
20
15
10

Figure 11. N-terminally 1xFLAG/GFP-tagged LIN-41 can fully rescue the *lin-41(n2914)* null phenotype

Worms expressing N-terminally 1xFLAG/GFP-tagged LIN-41 were crossed into *lin-41(n2914)* in order to assess if the tagged version of LIN-41 was functional. Furthermore, by performing RNAi against the *gfp* tag we reconfirmed the same. **A.** Panel ***a*** to ***c*** shows *lin-41(n2914)* worms rescued by the tagged *lin-41* transgene. ***c*****.** Shows 1xFLAG/GFP tagged LIN-41 expression in an oocyte marked by an arrow. Panel **(*d)*** to **(*f)*** shows the loss of rescue when we perform RNAi against the *gfp* tag in our tagged LIN-41. The worms appear dumpy ***(d)***, they are sterile ***(e)*** and we do not observe the transgene expression ***(f).*** Scale bar 20 um ***(a, d)*** and 50 um ***(b, c, e, f)***

B. Arrows mark the somatic expression of tagged LIN-41 observed during the larval stage 3 worms. Bright florescent dots are gut granules auto fluorescing, represented by brackets. In the adult stage worms, we observe germline expression. Arrow heads mark perinuclear localization of LIN-41. LIN-41 is localized in the cytoplasm and showed a granular distribution 40x magnification.

C. 1xFLAG/GFP tagged LIN-41 is immunoprecipitated through an anti FLAG antibody. Elution of LIN-41 was performed by competing with FLAG peptide. 0.25 % of input, 0.29 % of unbound and 2.5 % of IP were loaded, respectively.

5.2.2. LIN-41 protein interacts with *lin-29* mRNA

After generating a tagged version of LIN-41, we turned our attention to assessing its function. Several recent studies have revealed that LIN-41 can not only silence mRNA but also drive protein ubiquitylation (Rybak et al., 2009; Chang et al., 2012; Chen et al., 2012; Loedige et al., 2012). The question was, which of these mechanisms were involved in the regulation of LIN-29. The initial hints came from multiple reports that have established the NHL domain playing an important role in mediating direct binding of TRIM-NHL proteins to mRNA (Chen et al., 2012; Loedige et al., 2012; Loedige et al., 2014). LIN-41 contains six NHL repeats near the C-terminal end of the protein (Slack et al., 2000), which suggested LIN-41's ability to recruit target mRNAs. Moreover, additional evidence from Tocchini et al (2014) suggested that LIN-41's RING domain does not confer it with the ability to ubiquitylate protein, thus arguing against the possibility of LIN-29 protein regulation by ubiquitin-mediated protein degradation. All these evidence further supported hypothesis of LIN-41 mediated translational regulation of *lin-29*.

Yet the actual evidence of LIN-41 binding to the *lin-29* mRNA was still lacking. Hence we set out to investigate LIN-41's mRNA binding potential. By using co-immunoprecipitation (Co-IP) we analyzed and compared the eluates from worms expressing transgenic N-terminally 1xFLAG/GFP tagged LIN-41 to our control worms expressing 1xFLAG/GFP tagged SART-3 (Fig. 11C) (Ruegger et al., 2015) as well as the N2 strain. By performing RT-qPCR, we assessed the enrichment of *lin-29* mRNA in LIN-41 IP and found it to be highly enriched when compared to the control mRNAs such as *actin* and *unc-54* (Fig. 12).

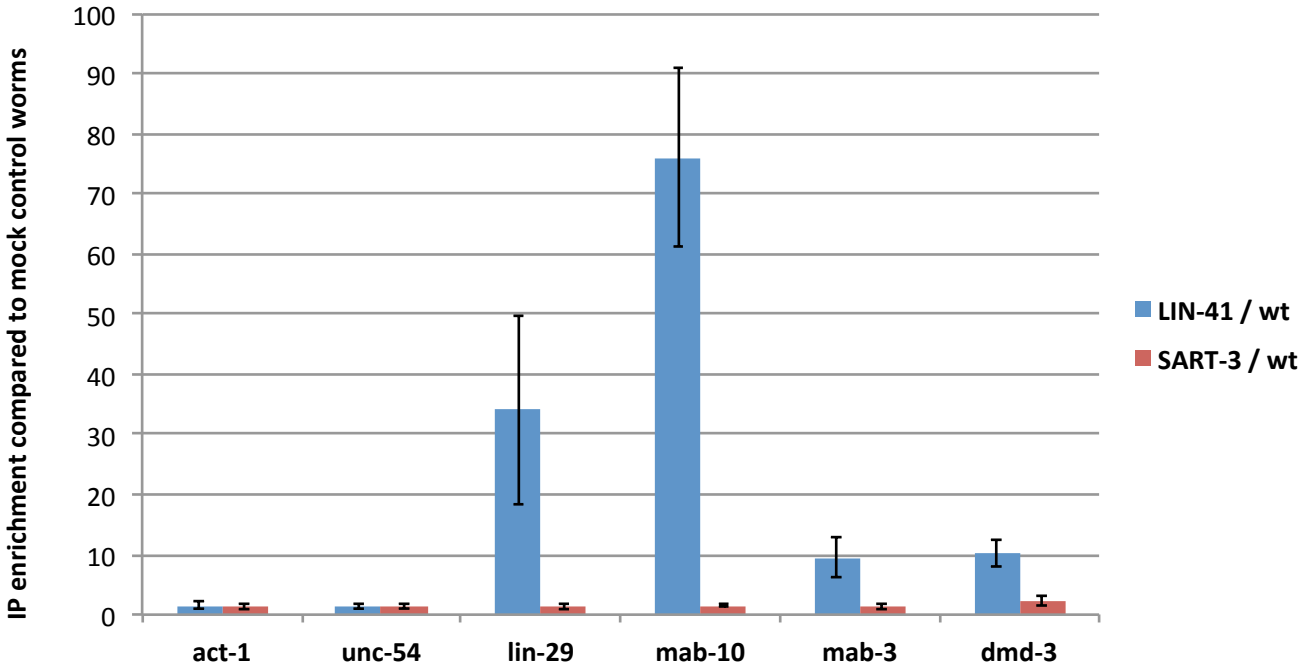

IP enrichment compared to mock control worms
100
90
80
70
60
50
40
30
20
10
0
act-1
unc-54
lin-29
mab-10
mab-3
dmd-3
LIN-41 / wt
SART-3 / wt

Figure 12. LIN-41 binds to the mRNAs of *lin-29, mab-10, mab-3* and *dmd-3*

RT-qPCR analysis revealed enrichment of putative LIN-41 targets *lin-29, mab-10, mab-3, dmd-3* (Aeschimann unpublished data) getting enriched in FLAG tagged LIN-41 IP relative to our control IPs performed using worms expressing FLAG tagged SART-3 and N2 worms. Control mRNAs *actin* and *unc-54* are not enriched in LIN-41 IP. Error bars indicate SEM from triplicate experiment.

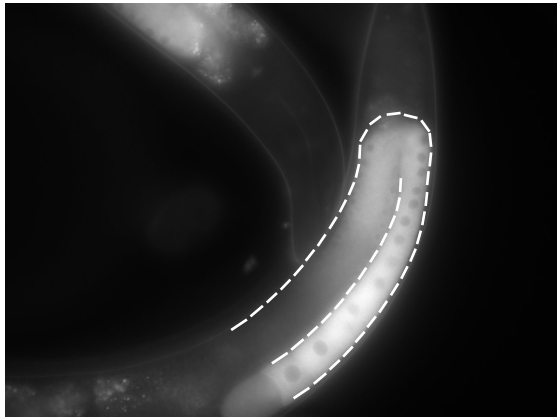
5.2.3. LIN-41 mediated regulation of LIN-29 expression occurs at translational level by binding to the 5'UTR region

With the evidence in hand that LIN-41 does indeed bind to *lin-29* mRNA, we wanted to investigate to which region in the *lin-29* mRNA would LIN-41 protein bind? Research has revealed that the untranslated region of an mRNA are usually the hotspots that contain regulatory sequences to which the trans-acting factors such as RNA binding proteins are known to bind (Glisovic et al., 2008). In fact translational control mechanisms often involve interactions between the 3' or 5' untranslated regions (UTRs) of mRNA and RNA-binding proteins (Barrett et al., 2012; Khosrow 2011). Furthermore, it is well established that many proteins binding to the 3' UTR elements can influence the fate of an mRNA in several ways, which include translation regulation, degradation, and localization of an mRNA (Mazumder et al., 2001). Consequently we began with the assumption that the 3'UTR region in *lin-29* mRNA could possibly contain the potential binding sites for LIN-41 protein. In order to test our hypothesis, we created a reporter worm line expressing *gfp* and included the *lin-29* 3'UTR region. The reporter was driven by the ubiquitously and constitutively active *dpy-30* promoter, which would allow us to test LIN-41 mediated regulation of *lin-29* 3'UTR in multiple tissues simultaneously.

Interestingly the most apparent change in the levels of the *lin-29* 3'UTR reporter expression was observed in the oogenic germline, where LIN-41 protein is expressed in adult worms, starting from the late pachytene stage and continuing its expression strongly in the oocytes (Fig. 13A). This was surprising since *lin-29* mRNA does not appear to be expressed in the germline (Reinke et al., 2004;Tocchini et al., 2014). Nonetheless, a strong inverse correlation between the expression of *lin-29* 3'UTR reporter and LIN-41 expression was observed. The GFP signal was rapidly down-regulated in the region of the gonad where LIN-41 was expressed (Fig. 13B). Strikingly our control worms containing an unregulated *unc-54* 3'UTR did not show any repression in the regions of the gonad where the LIN-41 protein was expressed (Fig. 13C). Moreover, upon RNAi against *lin-41*, we also observed de-repression of the *lin-29* 3'UTR reporter in the region of the gonad where we usually observe *lin-41* mediated down-regulation (Fig. 14).

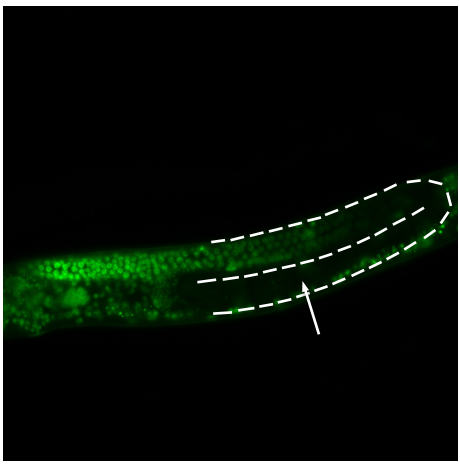
A

lin-41(n2914); Plin-41::flag::gfp::lin-41::lin-41 3'UTR



B

Pdpy-30::gfph2bpest::lin-29 3'UTR



C

Pdpy-30::gfph2bpest::unc-54 3'UTR

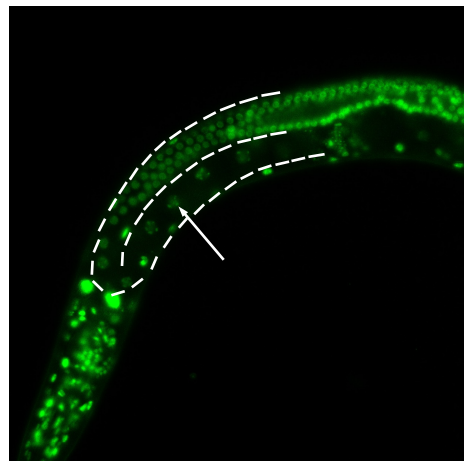
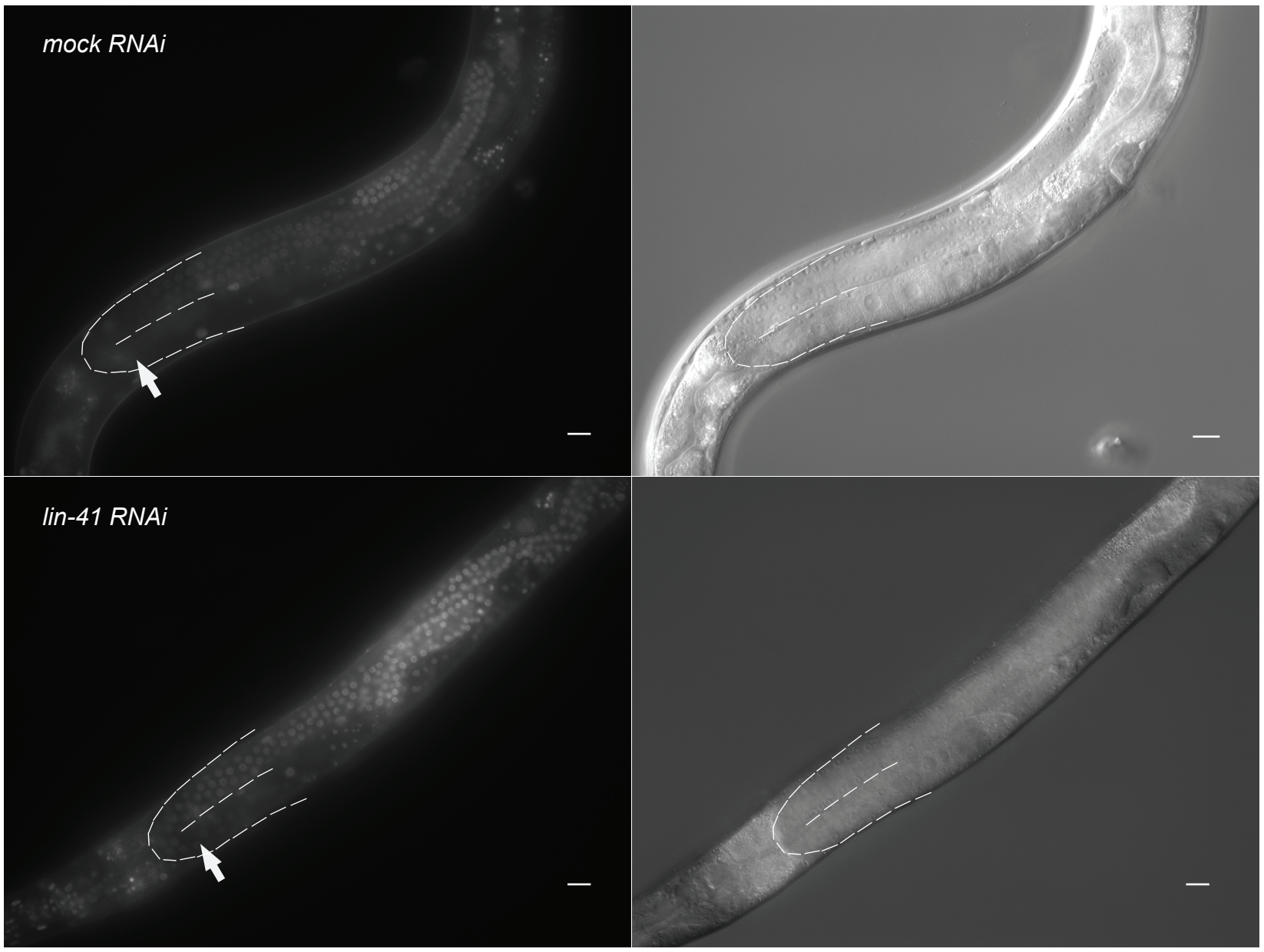


Figure 13. *lin-29* 3'UTR results in repression of the reporter in the proximal germline .

A. Expression of 1xFLAG/GFP tagged LIN-41 in the germline from late pachytene stage to the oocytes. **B.** A *gfp* reporter driven by the *dpy-30* promoter and containing *lin-29* 3'UTR is highly expressed in the distal part of the gonad where there is no LIN-41 expression but resulted in strong down- regulation in the proximal germline where LIN-41 is strongly expressed in the adult worms (arrow). **C.** Control worms containing *gfp* reporter driven by the same promoter however, consisting of an unregulated *unc-54* 3'UTR displays no such down-regulation in the germline (arrow).

A


Pdpy-30::gfph2bpest::lin-29 3'UTR
mock RNAi
lin-41 RNAi

B

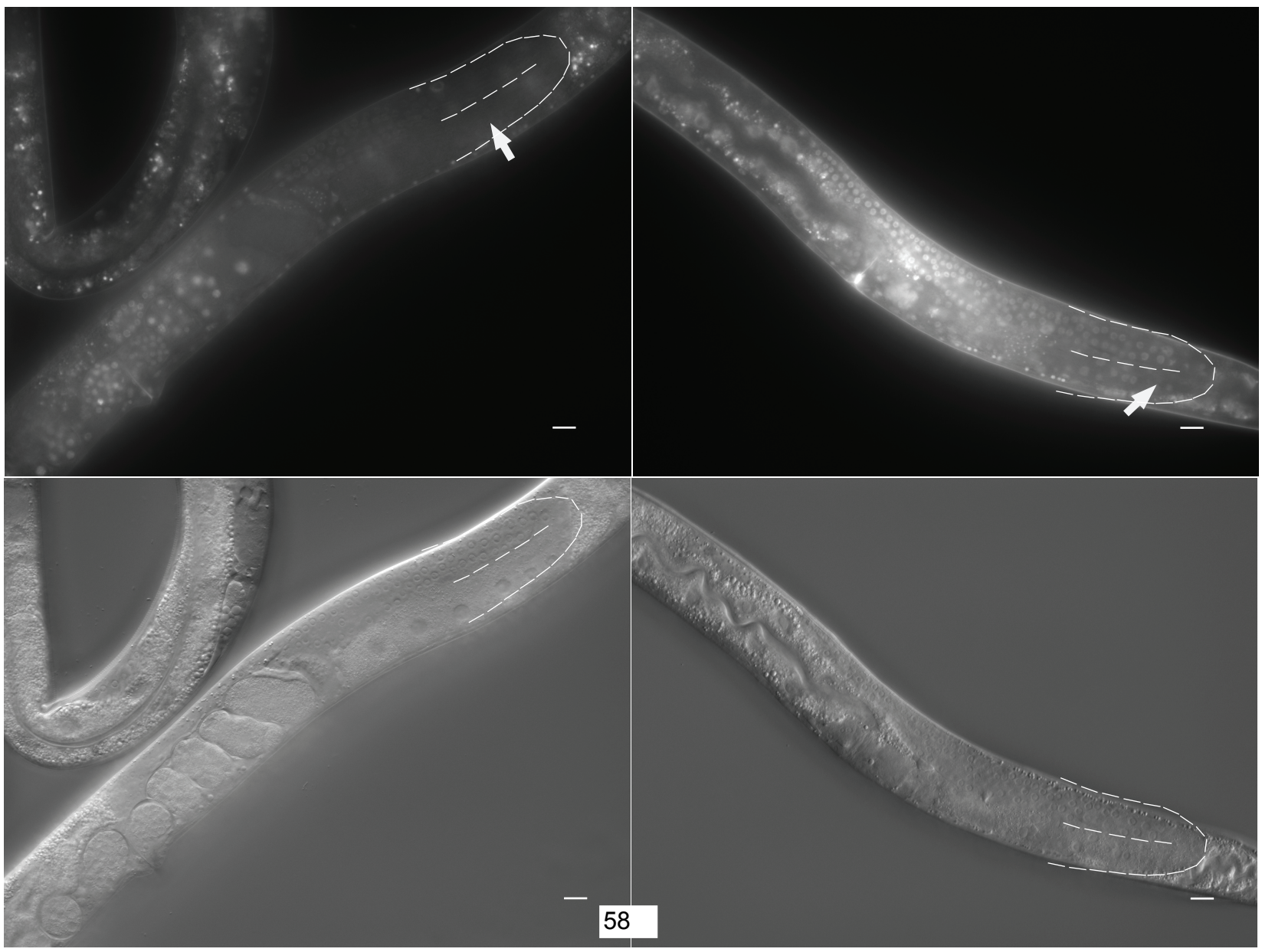

Pdpy-30::gfph2bpest::lin-29 3'UTR
lin-41(n2914); Pdpy-30::gfph2bpest::lin-29 3'UTR

Figure 14. Knockdown of *lin-41* results in up regulation of *lin-29* 3'UTR reporter in the germline .

A. Upon *lin-41* RNAi, the reporter containing *lin-29* 3'UTR is de-repressed in the region from late pachytene stage to the oocytes, marked by arrow. Of note knock down of *lin-41* resulted in abnormal germline. In the same region of the germline the *lin-29* 3'UTR reporter continued to be repressed when the worms were treated with mock RNAi, marked by arrow.

B. Upon crossing our *lin-29* 3'UTR reporter worms into *lin-41(n2914)* worms, we observed similar albeit stronger up regulation of the reporter as compared to the worms on *lin-41* RNAi. Scale bar indicates 50 um.

Pdpy-30::gfph2bpest::lin-29 3'UTR

L3 stage worms

L4 stage worms

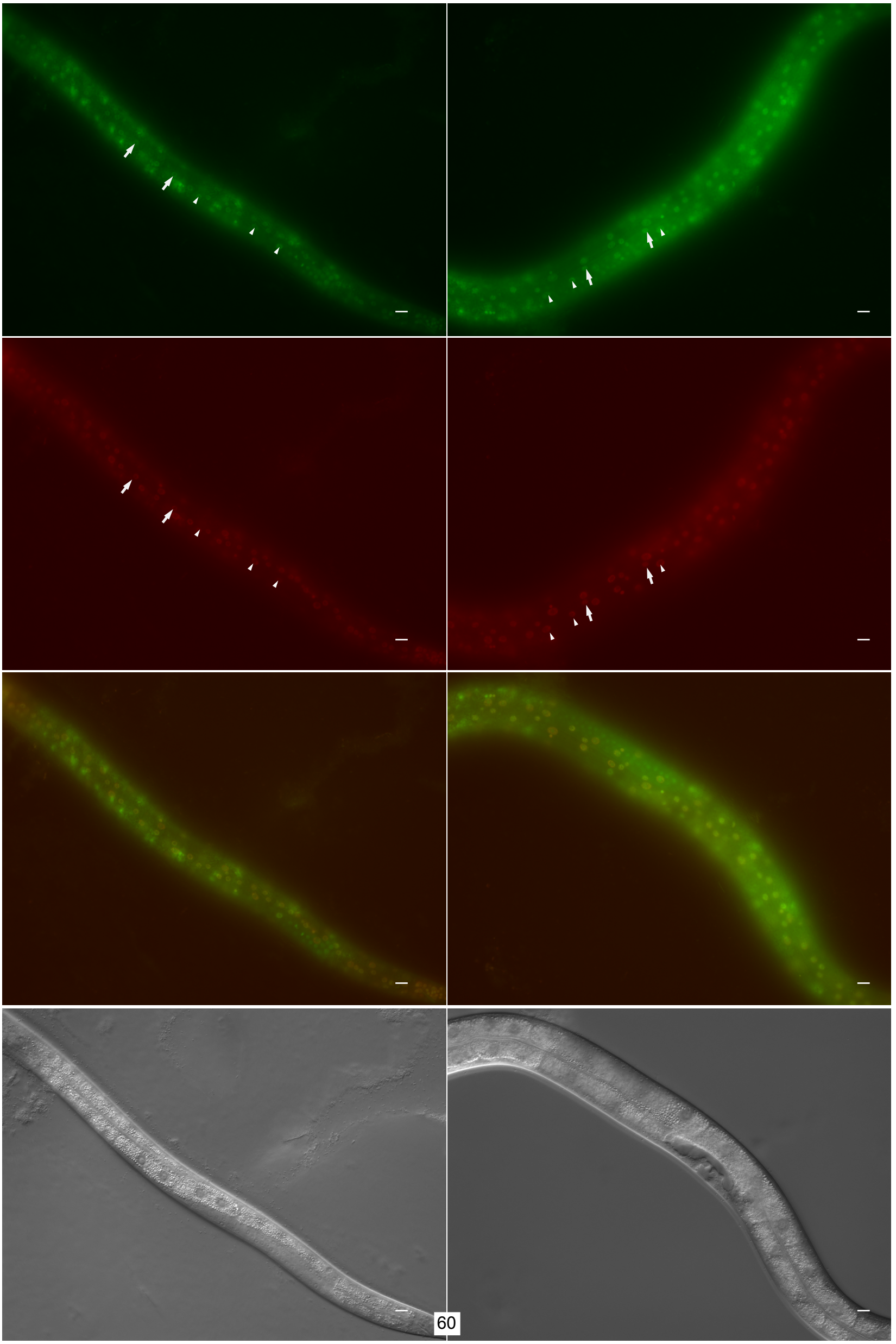


Figure 15. *lin-29* 3'UTR reporter doesn't seem to be regulated in the hypodermis.
Comparison of the expression levels of *lin-29* 3'UTR reporter between larval stage 3 worm and larval stage 4 worm. *lin-29* 3'UTR reporter expression does not vary between these two developmental stages of the worm in the hypodermis. Representative seam cells and hyp-7 cells marked by arrow and arrowhead respectively. Scale bar indicates 50 um.

In the hypodermis LIN-29 protein expression is required for larval-to-adult transition and although the *lin-29* mRNA starts getting expressed from larval stage 2, the LIN-29 protein is expressed only from late larval stage 4 and beyond i.e. when LIN-41 protein levels go down (Ambros and Horvitz, 1984; Bettinger et al., 1996; Slack et al., 2000). Hence we wondered if we could observe any changes in the reporter level in hypodermis. We observed overall weak expression of the *lin-29* 3'UTR reporter as compared to the control reporter containing *unc-54* 3'UTR. However, this weak expression did not change between larval stage and adult stage worms over the development of the worm. This finding surprised us, since we were expecting up-regulation of the *lin-29* 3'UTR reporter starting from larval stage 4 when LIN-41 protein levels start to reduce, thus releasing the repression over the reporter (Fig. 15). Furthermore, we also failed to see any significant up-regulation of the *lin-29* 3'UTR reporter upon *lin-41* RNAi. To investigate the possibility of regulation occurring either through the *lin-29* promoter or the 5'UTR, we created a reporter driven by the *lin-29* promoter and also containing the 5'UTR region (Fig. 16). This change resulted in a very clear phenotype observed in the hypodermis. We observed that the *gfp* reporter driven by *lin-29* promoter + 5' UTR and fused to *lin-29* 3'UTR resulted in a very weak reporter expression until early larval stage 4. From larval stage 4 onwards as *let-7* miRNA starts down regulating LIN-41 protein expression (Slack et al., 2000), we observed increase in the *lin-29* reporter expression (Fig. 17A). Furthermore, by performing RNAi against *lin-41,* we also observed precocious reporter expression starting from larval stage 2 onwards, thus verifying that indeed LIN-41 mediates repression of the *lin-29* reporter (Fig. 17B). Upon analyzing our control reporter driven by *lin-29* promoter and containing unregulated heterologous *unc-54* 3'UTR, we observed identical repression pattern to our reporter driven by *lin-29* promoter and containing *lin-29* 3'UTR (Fig. 18A & 18B). Collectively all these results suggested that the *lin-29* 3'UTR was dispensable for LIN-41 mediated regulation and indicating a mode of regulation that occurs either through the promoter by transcription and/or 5'UTR region.

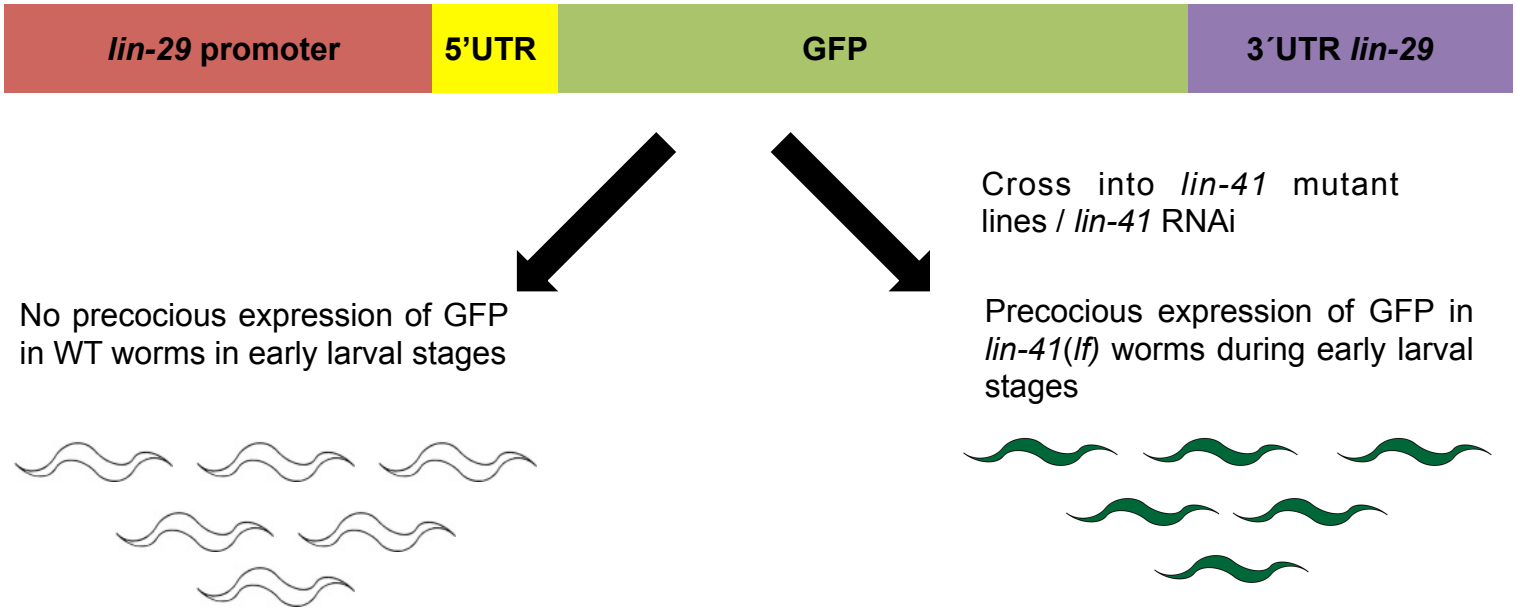
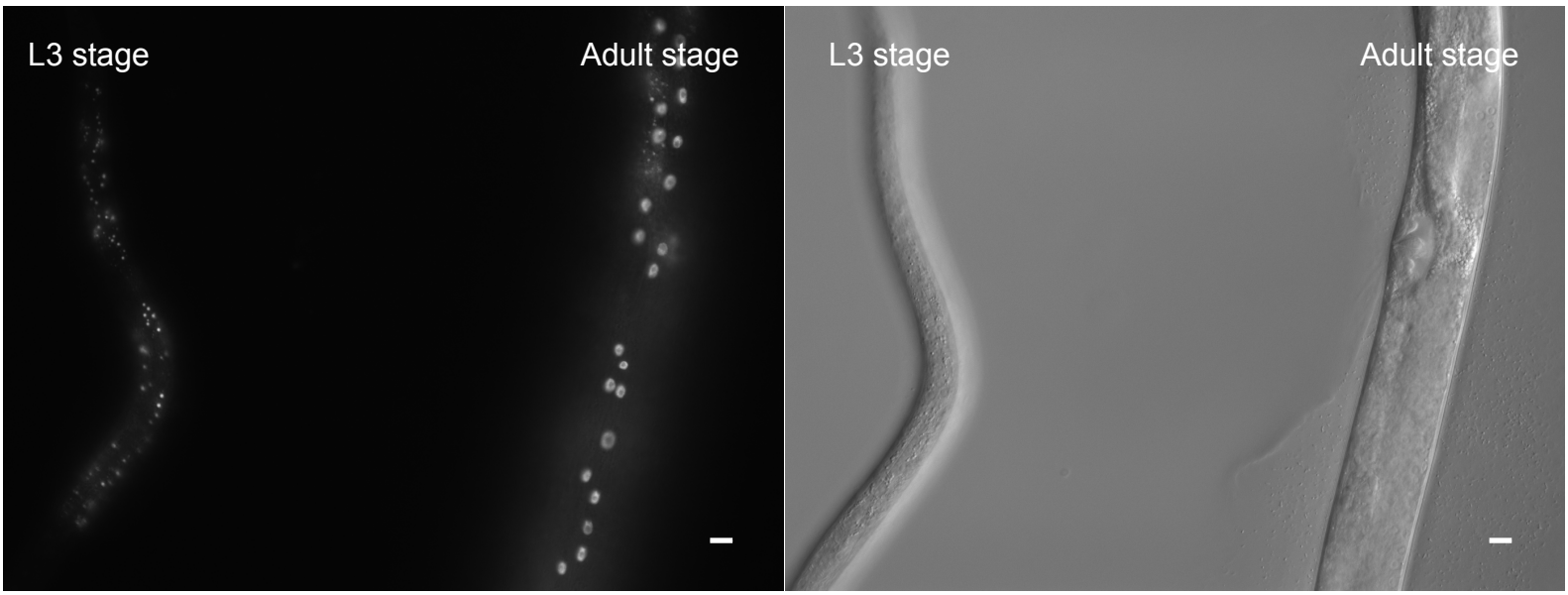


Figure 16. *lin-29* reporter construct
Schematic depiction showing the *lin-29* reporter construct containing *gfp* driven by *lin-29* promoter and containing 5' UTR as well as the 3'UTR region of *lin-29*
LIN-41 protein represses LIN-29 protein expression. WT worms should display no GFP expression during early larval stages if LIN-41 regulates the *lin-29* reporter expression. In absence of LIN-41, precocious expression of the GFP should be observed during early larval stages due to loss of repression over *lin-29* reporter.

Plin-29::gfph2bpest::lin-29 3'UTR

A



B

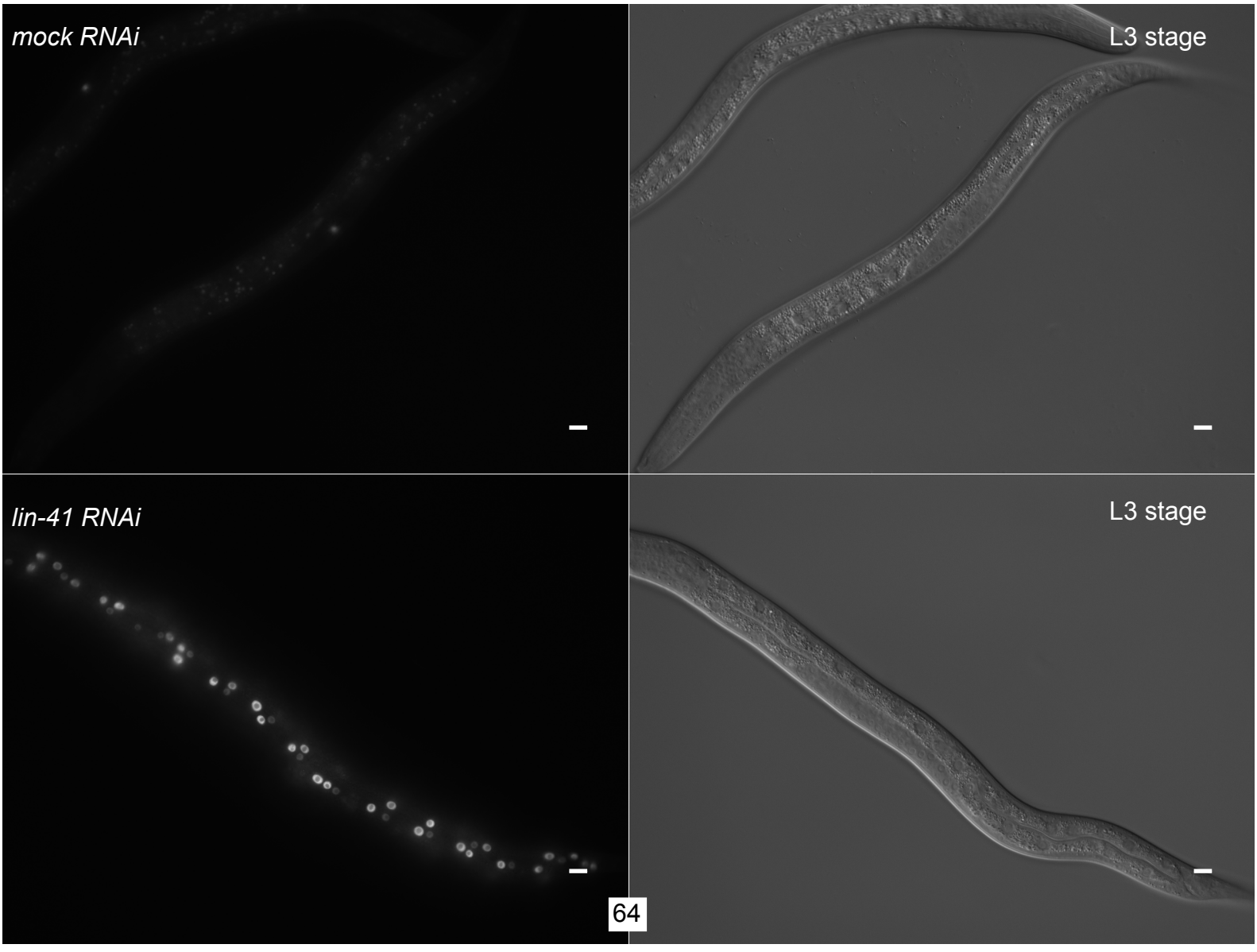
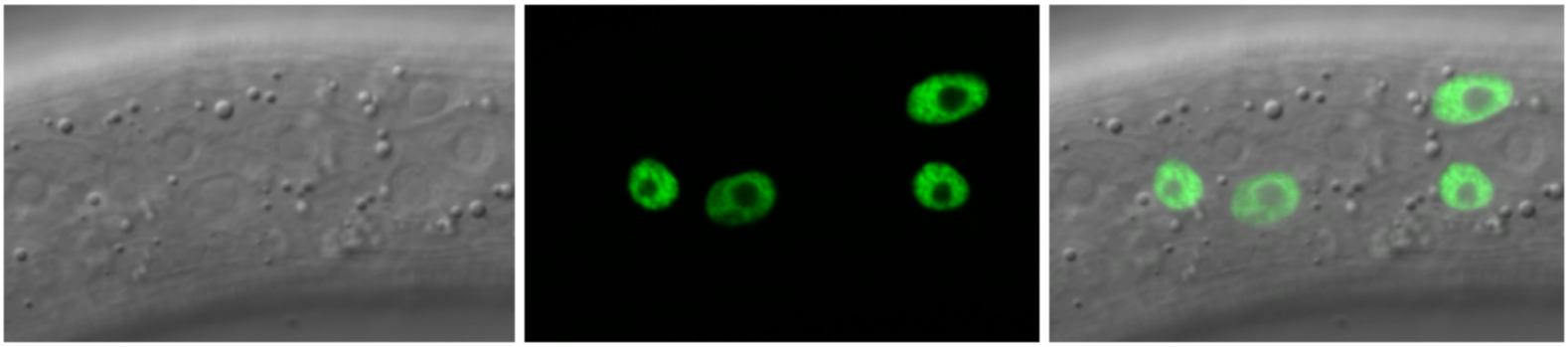


Figure 17. *lin-29* promoter + 5'UTR based *lin-29* 3'UTR reporter results in strong LIN-41 mediated repression in hypodermis.
Replacing the *dpy-30* promoter in the *lin-29* 3'UTR reporter with the *lin-29* promoter + 5'UTR resulted in strong repression. **A.** Shows difference in reporter expression between a larval stage 3 worm versus an adult worm in the hypodermis.
B. RNAi against *lin-41* results in strong precocious up regulation of the reporter in larval stage 3 worms when compared to the mock RNAi. Scale bar indicates 50 um.

Plin-29::gfph2bpest::lin-29 3'UTR

A *mock RNAi*

lin-41 RNAi



Plin-29::gfph2bpest::unc-54 3'UTR

B *mock RNAi*

lin-41 RNAi

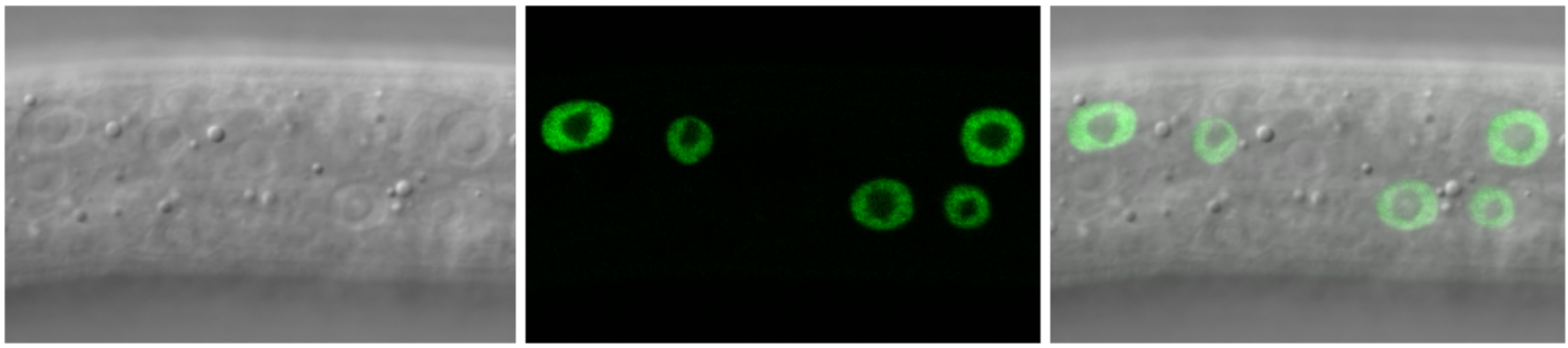


Figure 18. *lin-29* promoter containing the 5'UTR region results in down regulation mediated by LIN-41

Upon changing the promoter from *dpy-30* to *lin-29*, we observed strong repression of the reporter in the hypodermis. A. Shows the reporter remains repressed under mock RNAi conditions. RNAi against *lin-41* resulted in up regulation of the reporter in hypodermis.

B. Shows worms expressing *gfp* reporter driven by the *lin-29* promoter and containing the 5'UTR region, however, the *lin-29* 3'UTR is replaced by *unc-54*. The reporter containing an unregulated *unc-54* 3'UTR continue to show identical repression pattern to *lin-29* 3'UTR reporter suggesting that an element in the promoter region is responsible for the reporter regulation by LIN-41 protein.

The possibility of transcription-based regulation of our reporter was unlikely considering the observation in the previous studies, which indicate that *lin-29* mRNA transcript is expressed from early larval stages (Rougvie and Ambros 1995; Bettinger et al., 1996). Incidentally a parallel experiment of Ribosome profiling analysis performed by another Ph.D. student in our lab, Florian Aeschimann demonstrated that indeed LIN-41 mediates LIN-29 regulation at translational level and not by transcription, when he compared changes in ribosome protected mRNA fragments of *lin-29* to the mRNA levels of *lin-29*. Further analysis revealed that a 200 base pair 5'UTR region in the *lin-29* mRNA transcript was responsible for LIN-41-mediated translation inhibition and indeed when he deleted this 200 base pair region from the *lin-29* promoter, we observed de-repression of the reporter (F. Aeschimann unpublished data).

Moreover, the ribosome profiling analysis also revealed changes in the levels of additional mRNA targets, suggesting the possibility of LIN-41 mediated regulation. Accordingly we assessed for enrichment of these putative targets using co-immunoprecipitation and compared the eluates from worms expressing transgenic N-terminally 1xFLAG/GFP tagged LIN-41 to our control worms expressing 1xFLAG/GFP tagged SART-3 (Ruegger et al., 2015) as well as the N2 strain. By performing RT-qPCR, we observed *mab-10, dmd-3* and *mab-3* getting enriched as compared to the control mRNAs- *actin* and *unc-54* (Fig. 12).

By performing LIN-41 Co-IP, we established that LIN-41 binds to the mRNA of *lin-29.* Moreover, we also could verify additional mRNA targets of LIN-41 that were found in the ribosome profiling analysis. Furthermore, the data from ribosome profiling and our reporter analysis demonstrated that *lin-29* regulation occurs at translational level and is mediated through the 5'UTR region in the *lin-29* mRNA.

5.2.4. Assessing how LIN-41 mediates translational regulation mechanistically

After establishing that LIN-41 indeed binds to *lin-29* mRNA in the 5'UTR region, thus resulting in its translational inhibition, we wondered, how LIN-41 would mechanistically function in order to carry out its role as a translational inhibitor. One approach, which could provide us with an answer to this question, was by assessing the potential protein-binding partners that may associate with LIN-41. In order to investigate the protein interaction partners of LIN-41, we turned to the worms expressing transgenic, N terminally 1xFLAG/GFP tagged LIN-41. As a control we utilized a worm strain expressing 1xFLAG/GFP tagged SART-3 (Ruegger et al., 2015), a nuclear RNA-binding protein of similar size to LIN-41. We carried out co-immunoprecipitations with lysates of these worms and subjected the eluates to mass spectrometry analysis.

Since LIN-41 is differentially expressed during the development of the worm, i.e. we observe somatic expression during larval stages and predominantly germline expression in the adult stage, we performed two different IPs that enabled us to assess the stage specific protein-binding partners of LIN-41.
The first LIN-41 IP was performed using mixed stage worm culture, where the expressed LIN-41 was predominantly originating from the germline. For assessing the LIN-41 protein-binding partners in soma, we collected larval stage 3 worms when LIN-41 is maximally expressed in somatic cells. Eluates from both IPs were subjected to mass spectrometry analysis (MS) and the proteins identified in LIN-41 IP but not in the SART-3 control IP were considered as potential binding partners of LIN-41. Interestingly both the IPs resulted in different set of proteins getting pulled down with *lin-41*. In the case of the mixed stage worm IP we observed multiple proteins, which are listed in Supplementary Fig.1.1- 1.3. We observed hits such as CGH-1, CAR-1 and SMG-2 that are involved in post-transcriptional RNA regulation and known to be associated with P granules as well as other cytoplasmic RNA granules (Eulalio et al., 2007; Noble et al., 2008; Pitt et al., 2000, Minshall et al., 2009; Page et al., 1999).

Assessing protein-binding partners of LIN-41 in somatic tissue was technically more challenging due to the fact that LIN-41 protein is very weakly expressed. In order to circumvent the problem we had to augment the Co-IP experiment with large quantity of larval stage 3 worms. The analysis revealed eight proteins clearly getting enriched in the LIN-41 IP as compared to SART-3 IP control listed in (Supplementary Fig. 1.4).

Strikingly several protein interaction partners that we found in LIN-41 Co-IP are implicated in RNA regulation. We wanted to assess if these protein hits

were true interaction partners of LIN-41 and involved in LIN-41 mediated translational regulation of mRNAs. In order to investigate this, we decided to use our *lin-29* reporter line as a sensor. We wondered if RNAi against putative protein interaction partners of LIN-41 could result in either precocious GFP expression in larval stage 3 worms or conversely result in lack of up-regulation of the reporter in larval stage 4 and beyond. We tested multiple Co-IP hits that we thought could possibly play an important role in mediating translational regulation, in addition to the proteins that are generally involved in miRNA mediated translational inhibition (Mathys et al., 2014), listed in (Supplementary Table. 1). Furthermore, we also expanded our criteria and performed RNAi against 200 plus *let-7* bursting phenotype suppressors that we published in Rausch et al (2015) paper. Surprisingly none of these factors upon down-regulation by RNAi resulted in precocious GFP expression similar to what we observed for *lin-41* RNAi.

Interestingly upon *laf-1* RNAi, the *lin-29* reporter failed to express in the larval stage 4 worms and beyond, when the LIN-41 mediated repression ends (Fig. 19A). This observation did put forth an interesting possibility of LIN-41 blocking LAF-1 activity, which may be required to promote *lin-29* reporter expression. Previous studies have indicated that *laf-1* gene codes for a putative DEAD-box RNA helicase and is required for viability of the worms (Hubert and Anderson, 2009). Of note, *laf-1* loss-of-function is lethal for the worms. Indeed the worms grown under *laf-1* RNAi conditions were sick, displaying strong developmental delay and many worms failed to enter adult stage. Initially we thought that this lack of *lin-29* reporter up-regulation in the larval stage 4 worms and beyond was due to sickness observed in the worms as well as the developmental delay caused due to *laf-1* RNAi. However, upon testing the control reporter consisting of an unregulated *unc-54* 3'UTR driven by *dpy-30* promoter under *laf-1* RNAi, we did not observe similar down regulation of the reporter in these control worms, which suggested that lack of up regulation observed in the *lin-29* reporter, was specific (Fig. 19B). In order to analyze this *laf-1* RNAi induced *lin-29* reporter phenotype further, we crossed the *lin-29* reporter worms in *lin-41(ma104). lin-41(ma-104)* is a *lin-41* hypomorh, which resulted in precocious up-regulation of the *lin-29* reporter. We then tested if the precocious up regulation of the *lin-29* reporter in *lin-41(ma104)* worms was reversed due to *laf-1* RNAi. The rational behind this experiment was, if LAF-1 protein is indeed required for the up-regulation of the reporter expression, then knocking down *laf-1* should result in similar loss of up-regulation of the precociously expressed *lin-29* reporter in *lin-41(ma104)* background. However, upon assessing these worms under *laf-1* RNAi, we observed no change in the precocious expression of the *lin-29* reporter (Fig. 19C), thus suggesting that although *laf-1* is an interesting candidate found in LIN-41 Co-IP, it may be a false positive.

A

Plin-29::gfph2bpest::lin-29 3'UTR

mock RNAi

laf-1 RNAi

B

Pdpy-30::gfph2bpest::unc-54 3'UTR

mock RNAi

laf-1 RNAi

C

lin-41 (ma104); Plin-29::gfph2bpest::lin-29 3'UTR

mock RNAi

laf-1 RNAi

Figure 19. *lin-29* reporter down regulation by *laf-1* RNAi is non-specific
A. *lin-29* reporter remained repressed in the worms grown under *laf-1* RNAi even in larval stage 4 and beyond. *Mock* RNAi does not display this phenotype.
B. Control reporter containing *unc-54* 3'UTR and driven by *dpy-30* promoter does not display repression at any stage of the worm under *laf-1* RNAi.
C. The top image in the panel C shows precocious expression of *lin-29* reporter crossed into *lin-41(ma104)* mutant worms during the larval stage 3 under *mock* RNAi. Knockdown of *laf-1* by RNAi did not result in repression of precociously expressed *lin-29* reporter in *lin-41 (ma104)* worms. Scale bar indicates 50 μm.

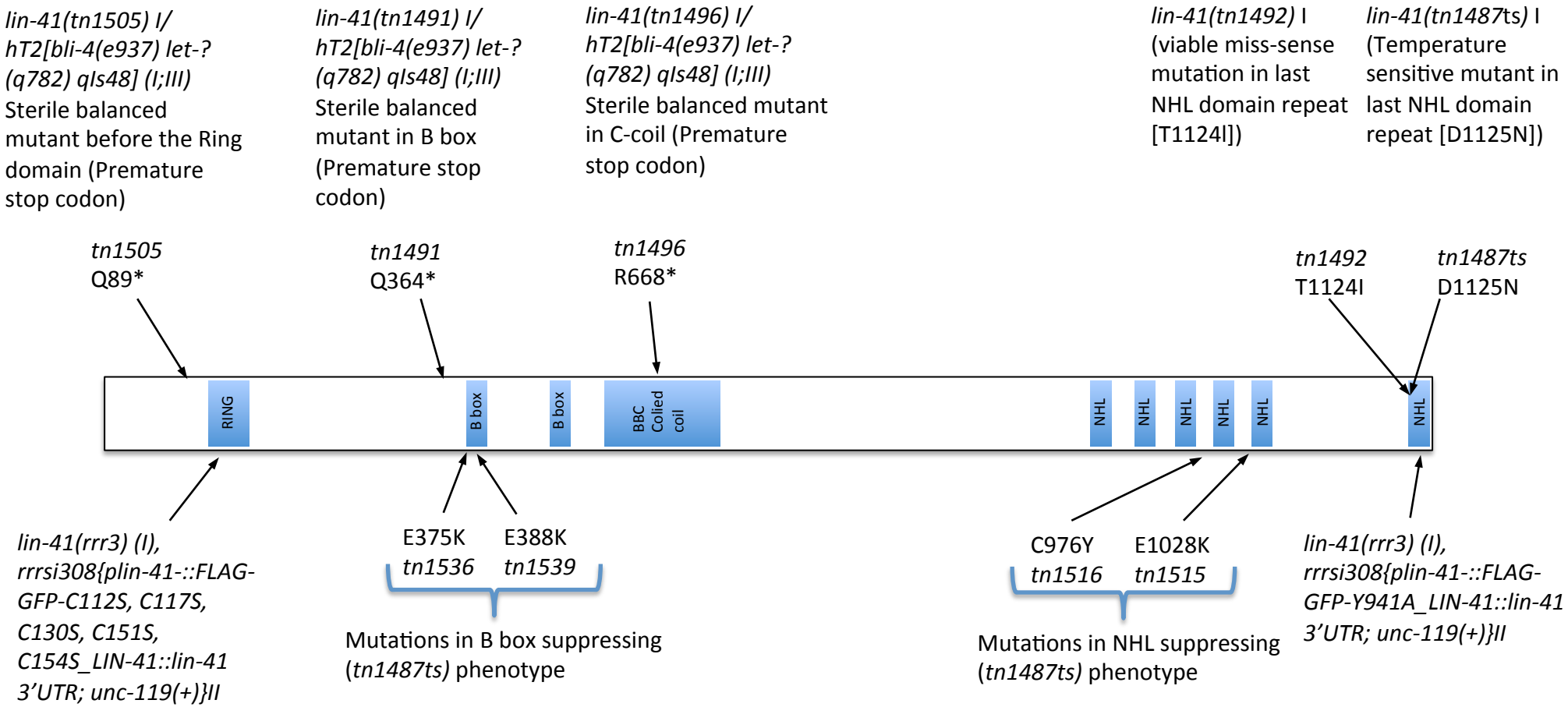


Figure 20. LIN-41 domain mutants
Schematic depiction of mutant strains that were used for analyzing the somatic phenotypes resulting from mutations in the individual domains of LIN-41.
* Represents mutants of LIN-41 that are consequence of a premature stop codon, resulting in either expression of truncated version of the protein or loss of expression resulting from Non-sense mediated decay.
lin-41(rrr3) (I), rrrsi308[plin-41-::FLAG-GFP-C112S, C117S, C130S, C151S, C154S_LIN-41::lin-41 3'UTR; unc-119(+)]II depicted simply as RING
lin-41(rrr3) (I), rrrsi308[plin-41-::FLAG-GFP-Y941A_LIN-41::lin-41 3'UTR; unc-119(+)]II depicted simply as NHL.

5.2.5. LIN-41 domain mutant analysis

Next we focused on the domain organization of LIN-41 to see if it could provide us with insight into the functioning of the protein. LIN-41 belongs to the TRIM-NHL protein family and has an intricate domain architecture consisting of a RING domain, two B-Boxes, a Coiled-Coil region, a Filamin domain and 6 NHL repeats (Slack et al., 2000). Each domain is implicated in different molecular activities, thus endowing LIN-41 with the possibility of functioning in multiple ways (Wulczyn et al., 2011). Furthermore, this observation does put forth many questions such as do these domains function together or independently? Does each domain have a specific function in individual tissues? And what would be the resultant phenotypes if we were to mutate individual domains in LIN-41. Incidentally *lin-41* mutants that were available from work carried out in two published papers- (Tocchini et al., 2014 and Spike et al., 2014) (Fig. 20 & Table.1) allowed us to investigate LIN-41 domain function.

In contrast to the work published in these two papers, which focused on the regulatory role of LIN-41 in the germline, we were specifically interested in dissecting the function of LIN-41 and its individual domains in somatic tissues. In the soma a clearly analyzable phenotype that can be observed upon *lin-41* loss-of-function as well as overexpression of *lin-41* gene, is its effect on the seam cells proliferation and their differentiation (Slack et al., 2000). In fact *lin-41* overexpression drives seam cells to continue cell division and remain in undifferentiated state. Conversely *lin-41* loss-of-function results in these seam cells to exit cell cycle and differentiate early during the development of the worm i.e. in the L3 molt. By assessing changes in this phenotype, we attempted to study the effects of LIN-41 domain mutants in the worm strains.

From the Spike et al (2014) paper, we analyzed a total of nine worm strains. Out of nine mutant strains, three contained premature stop codon. These were *lin-41(tn1505)*, mutated in the RING domain at amino acid position 89 resulting in Q-> stop codon, [*lin-41(tn1491)*] having a mutation in the B-Box domain at amino acid position 364 resulting in Q->stop codon and Coiled-Coil domain mutant [*lin-41(*tn1496)] having a mutation at amino acid position 576 resulting in R-> stop codon. Whether these early stop codons result in expression of the truncated version of the LIN-41 protein or are complete null alleles is unclear, since our anti-LIN-41 antibody bound between Coiled-Coil and the Filamin domain, hence we were unable to test the protein expression. When counting worms showing early differentiation in seam cells due to loss of *lin-41* function, we found all three mutants exhibiting partial precocious alae at L3 molt.

Strain	% alae L3 molt (total count)	% worms expressing GFP at L3 molt (total count)
lin-41(tn1505) l/hT2[bli-4(e937) let-?(q782) qIs48] (I;III)	88% (25)	No data
lin-41(tn1491) l/hT2[bli-4(e937) let-?(q782) qIs48] (I;III)	96% (26)	100 % (25)
lin-41(tn1496) l/hT2[bli-4(e937) let-?(q782) qIs48] (I;III)	58% (25)	100 % (25)
lin-41 (tn1487tn1515) l (Intragenic suppressor mutant in NHL repeat)	0% (25)	100% (25)
lin-41 (tn1487tn1516) l (Intragenic suppressor mutant in NHL repeat)	0 % (25)	Extremely weak GFP(25)
lin-41 (tn1487tn1536) l (Intragenic suppressor mutant in B-Box)	4% (25)	100 % (25)
lin-41 (tn1487tn1539) l (Intragenic suppressor mutant in B-Box)	7% (28)	100 % (25)
lin-41(tn1487ts) l @25 C (Temperature sensitive)	10% (30)	100 % (25)
lin-41(tn1487ts) l @15 C (Temperature sensitive)	0 % (25)	100% (25)
lin-41 (tn1492) l Viable miss sense mutation	0% (25)	100% (25) after 31 hours (growth delay)
lin-41(rrr3) (I), rrrsi308[plin-41-::FLAG-GFP-C112S, C117S, C130S, C151S, C154S_LIN-41::lin-41 3'UTR; unc-119(+)]II	0% (25)	0% (25)
lin-41(rrr3) (I), rrrsi308[plin-41-::FLAG-GFP-B Box_LIN-41::lin-41 3'UTR; unc-119(+)]II	0% (25)	No data
lin-41(rrr3) (I), rrrsi308[plin-41-::FLAG-GFP-Y941A_LIN-41::lin-41 3'UTR; unc-119(+)]II	16% (25)	100% (25)

Table 1. Precocious phenotypes in *lin-41 (lf)* mutant strains
Assessing percentage of worms showing precocious alae defects as well as expression of *lin-29* reporter in L3 molt. Synchronized larval stage 1 worms were put onto OP-50 bacteria plates. All worms were grown at 25°C unless stated otherwise.

We observed that the mutant worms containing premature stop codon before the RING domain resulted in 83% worms displaying partial alae (n=25). In the case of stop codon in B-Box 99% worms displayed partial alae (n=25), where as for the Coiled-Coil domain stop codon, only 53% (n=25) showed partial alae (Table.1).

We also analyzed two mutants resulting from amino acid substitutions in the NHL domain. Interestingly the *lin-41(tn1487ts)* containing D->N amino acid substitution resulted in temperature-sensitive allele that caused sterility at non-permissive temperature (Spike et al., 2014). These worms are 100% fertile at 15 °C but completely sterile at 25°C. The *lin-41(tn1487ts)* demonstrated low penetrance of precocious alae formation with only 10% worms showing partial alae (n=30) (Table.1) in addition to the slight dumpiness at non-permissive temperature. In contrast *lin-41(tn1492)* due to the amino acid substitution at position 1124 (T->I) resulted in no worms showing any precocious alae formation (n=26) (Table.1). However, these worms displayed a developmental delay as well as low brood size in addition to the slight dumpiness.

In addition to the above-mentioned mutants, we also analyzed four intragenic suppressor mutants reversing the sterility and slight dumpy phenotype observed in the temperature sensitive mutant. Two suppressor mutations were located in B-Box domain at amino acid position 375 in *lin-41(tn1487 tn1536)* and 388 in *lin-41(tn1487 tn1539)*, both resulting into E->K amino acid substitution showed very low penetrance of precocious alae formation of 7 % (n=28) and 4% (n=25) respectively (Table.1). The remaining two suppressor mutations were in the NHL domain at amino acid position 976 in *lin-41(tn1487tn1516)* and 1028 in *lin-41(tn1487tn1515)*, resulting into C->Y and E->K amino acid substitution respectively and did not display any precocious alae formation (Table.1).

A. *Plin-29::gfph2bpest::lin-29 3'UTR*

B. *lin-41(tn1431); Plin-lin-29::gfph2bpest::lin-29 3'UTR*

C. *lin-41(tn1496); Plin-lin-29::gfph2bpest::lin-29 3'UTR*

D. *lin-41(tn1492); Plin-lin-29::gfph2bpest::lin-29 3'UTR*

E. *lin-41(tn1487ts); Plin-lin-29::gfph2bpest::lin-29 3'UTR*

F. *lin-41(tn1487tn1515); Plin-lin-29::gfph2bpest::lin-29 3'UTR*

G. *lin-41(tn1487tn1516); Plin-lin-29::gfph2bpest::lin-29 3'UTR*

H. *lin-41(tn1487tn1536); Plin-lin-29::gfph2bpest::lin-29 3'UTR*

I. *lin-41(tn1487tn1539); Plin-lin-29::gfph2bpest::lin-29 3'UTR*

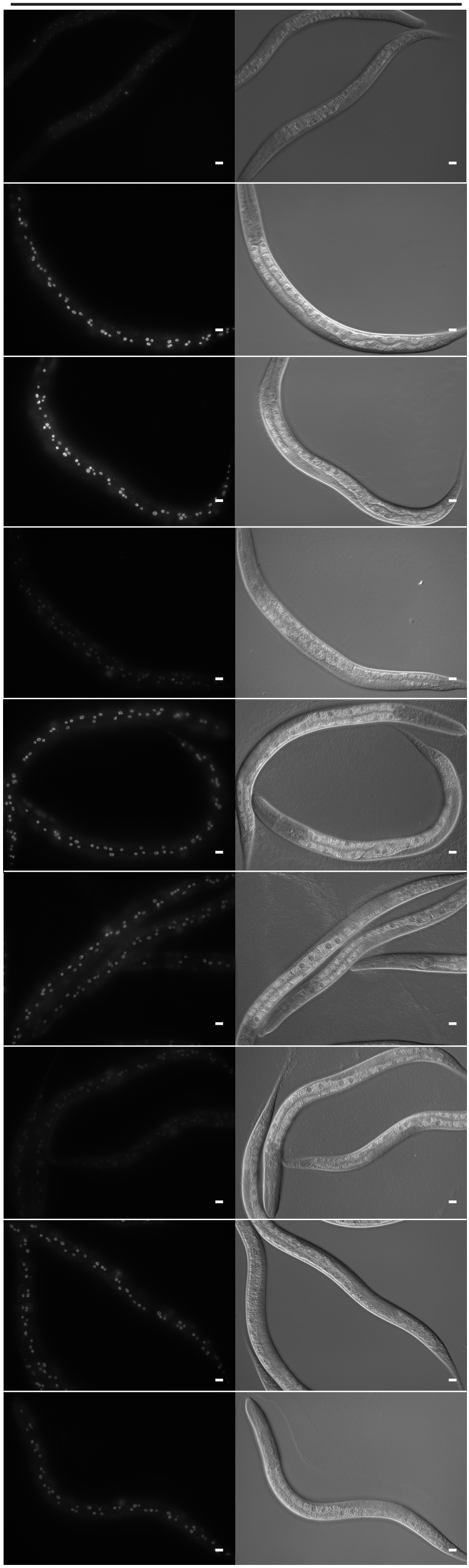
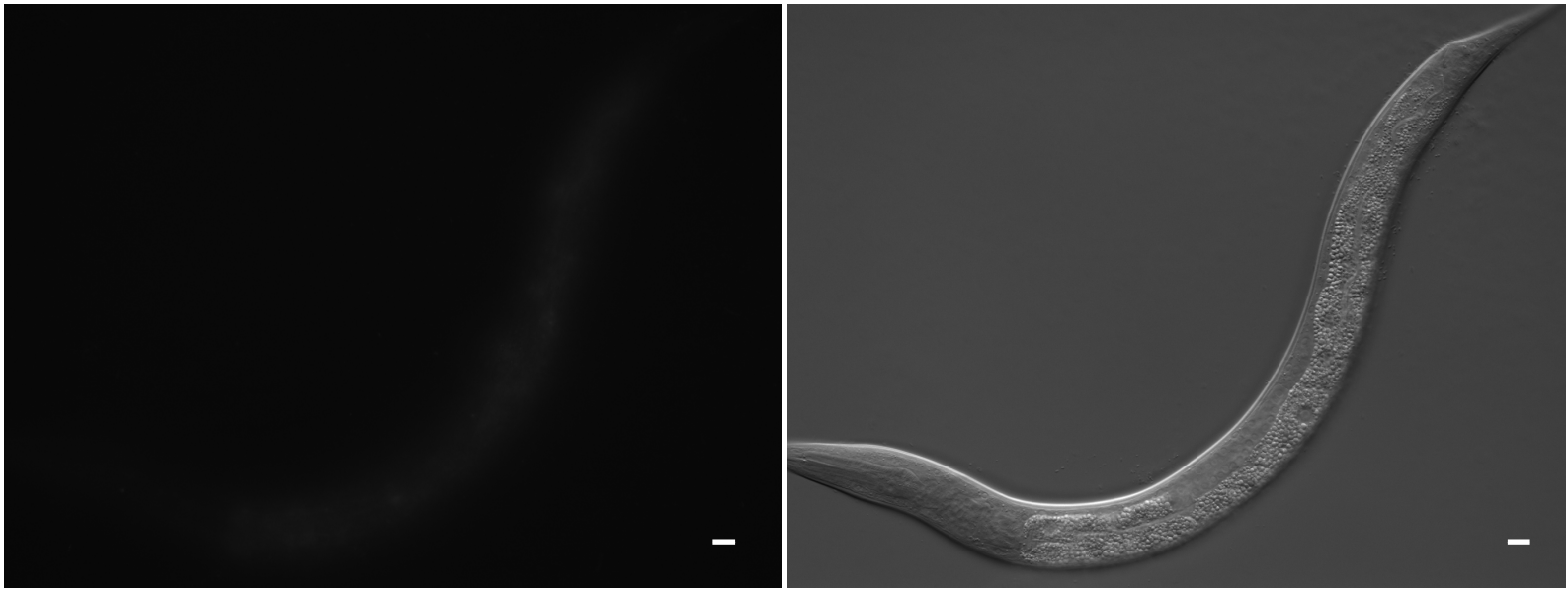


Figure 21. Mutations in LIN-41 domains result in varying degrees of precocious expression of *lin-29 reporter* .
A. WT control worms do not express *lin-29* reporter precociously at L3 molt .
(B and C). Strong precocious up regulation of the *lin-29* reporter was observed when crossed into *lin-41* mutants comprising of a premature stop codon in the B-Box domain and Coiled-Coil domain.
D. *lin-41(tn1492)* due to the amino acid substitution at position 1124 (T->I) in the NHL domain resulted in weak precocious up regulation of the reporter.
E. *lin-41(tn1487ts)* containing D->N amino acid substitution in the NHL domain resulted in strong precocious up regulation of the reporter.
(F and G). The suppressor mutation in *lin-41(tn1487tn1515)* the NHL domain resulted in modest reversal of precocious *lin-29* reporter expression, where as *lin-41(tn1487tn1516)* point mutant resulted in stronger reversal of the temperature sensitive mutant in NHL domain.
(H and I). Two suppressor mutations located in B-Box domain *lin-41(tn1487 tn1536)* and *lin-41(tn1487 tn1539)* rescued the temperature sensitive phenotype observed in *lin-41(tn1487ts)* in the germline, thus resulting in viable worms. However, the two suppressors could not reverse the somatic phenotype and as such we observed precocious expression of *lin-29* reporter. Scale bar indicates 50 um.

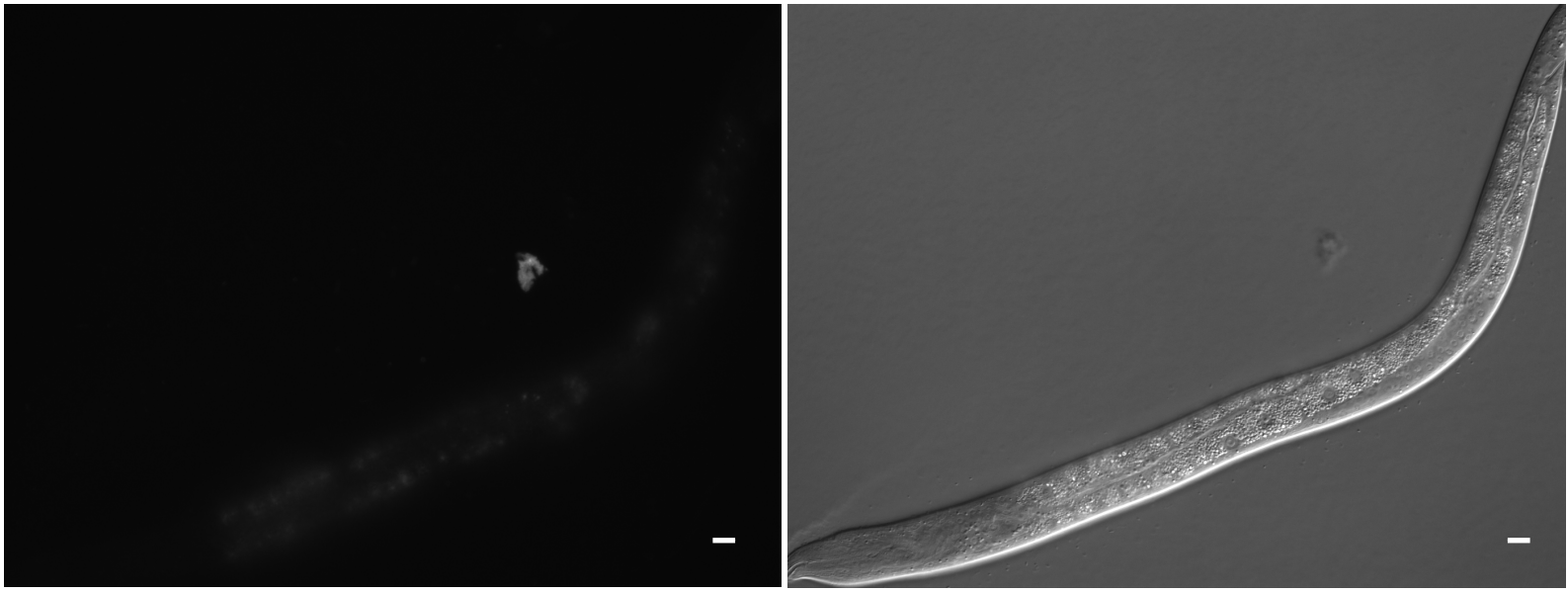
Next we crossed the *lin-29* reporter in the LIN-41 domain mutant backgrounds. The read out from the *lin-29* reporter enabled us to assess the role played by each domain in regulation of translation repression. The *lin-41* mutant resulting from early stop codon in the B-Box [*lin-41(tn1491)*] and Coiled-coil domain [*lin-41(*tn1496)] mutants produced strong up-regulation of the *lin-29* reporter (Fig. 21 B & C). In the case of the two NHL domain mutants, *lin-41(tn1487ts)* and *lin-41(tn1492)* resulted in moderate up-regulation of the reporter (Fig. 21 D & E). In the case of the suppressor mutations in the NHL domain, we observed modest reversal of precocious *lin-29* reporter expression in *lin-41(tn1487tn1515)* NHL domain suppressor mutant where as *lin-41(tn1487tn1516)* point mutant resulted in very weak *lin-29* reporter expression suggesting stronger reversal of the temperature sensitive mutant in NHL domain (Fig. 21 F & G). However, both B-Box intragenic suppressor mutants *lin-41(tn1487 tn1536)* and *lin-41(tn1487 tn1539)* were incapable of preventing the precocious *lin-29* reporter expression (Fig. 21 H & I).

In addition we also crossed the *lin-29* reporter into two LIN-41 domain mutants obtained from Tocchini et al (2014). Unlike the mutants from Spike et al (2014), the Tocchini et al (2014) mutants were created by mutating individual domains in the *gfp* tagged LIN-41 transgene. The mutant version of LIN-41 transgene was then integrated on chrII using MosSCI technique and crossed into *lin-41* null background worms. The advantage of analyzing these mutants due to the presence of a GFP tag was that we could track the expression of the mutant form of LIN-41 protein. The RING domain mutant was created by mutating four cysteine residues that are conserved. Upon crossing the *lin-29* reporter into the RING domain mutant, we did not observe any precocious *lin-29* reporter expression (Fig. 22B). Next mutant that we tested was resulting from a point mutation in the NHL domain at amino acid position 941 resulting in amino acid substitution from Y->A. We observed strong precocious up-regulation of the reporter in the NHL domain point mutant (Fig. 22C). Our attempt to cross our *lin-29* reporter line into the B-Box domain mutant failed due to technical reasons.

A *Plin-lin-29::mcherryh2bpest::lin-29 3'UTR*



B *lin-41(rrr3); Plin-41:flaggfplin-41(RING)::lin-41 3UTR; Plin-lin-29::mcherryh2bpest::lin-29 3'UTR*



C *lin-41(rrr3); Plin-41:flaggfplin-41(NHL)::lin-41 3UTR; Plin-lin-29::mcherryh2bpest::lin-29 3'UTR*

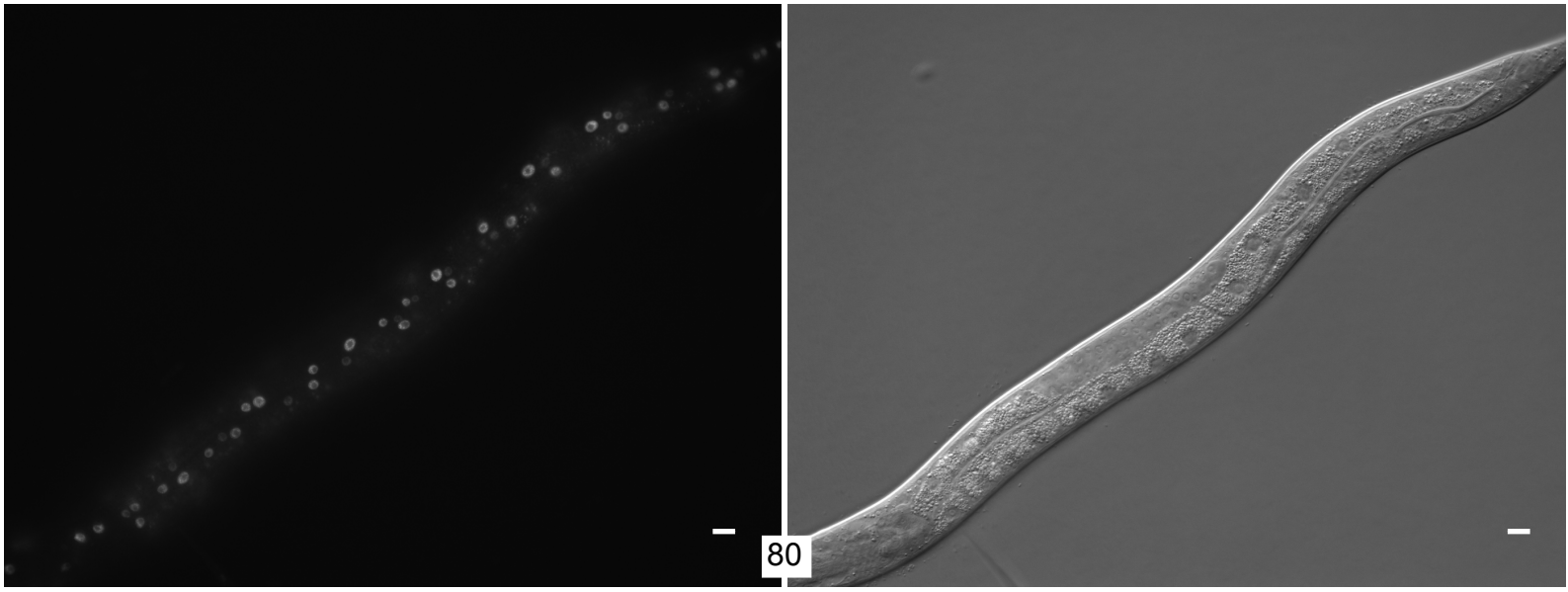


Figure 22. NHL domain but not the RING domain is important for regulation of *lin-29* reporter .

A. Control worms containing *mCherry* reporter driven by *lin-29* promoter and consisting of *lin-29* 3'UTR remains repressed in larval stage 3 worms.

B. The *mCherry::lin-29* 3'UTR reporter worms crossed into worms expressing LIN-41 containing mutations in the RING domain does not result in precocious up regulation of the reporter in larval stage 3.

C. Strong precocious up regulation of the *mCherry::lin-29* 3'UTR reporter is observed in the larval stage 3 worms upon crossing the reporter into worms expressing LIN-41 containing mutations in the NHL domain. Scale bar indicates 50 um .

6. Conclusion

The work carried out in the first part of this thesis was aimed at assessing whether *let-7* miRNA would regulate cell cycle regulators by directly binding to them. From the >40 cell cycle genes that were tested, we found two likely candidates *cdk-1* and *cdc-25.2* that could be potential *let-7* targets based on multiple criteria such as suppression of *let-7* bursting phenotype and reversal of seam cell over-proliferation and differentiation. However, the conclusion of this study was, *let-7* does not directly regulate *cdk-1* and *cdc-25.2* by binding to their 3'UTR regions. Our analysis also revealed that the downstream effector LIN-29 with its transcription co-factor MAB-10 modulates this regulation (Fig. 24).

In continuation of the first project, we revealed how *let-7's* major down-stream target LIN-41 regulates its target i.e. *lin-29*. Our results revealed that LIN-41 binds to *lin-29* mRNA, thus regulating LIN-29's protein levels at translational level. Further study showed that this regulation is mediated by LIN-41 protein binding to the 5'UTR region of *lin-29* mRNA. Moreover, the ribosome profiling analysis revealed additional mRNA targets of LIN-41, which we verified using LIN-41 immunoprecipitation experiment. Although our attempt to assess the protein-interaction partners of LIN-41 resulted in a potentially interesting list of candidates, at present we could not confirm if the proteins in our list are genuine interactors of LIN-41. Lastly the LIN-41 domain mutant analysis in combination with our *lin-29* reporter assay does raise a question about why *lin-29* reporter expression does not correspond to alae formation.

7. Discussion

Spatio-temporal control of gene expression is critical for the proper development of an organism. One such pathway that has significantly enhanced our understanding of how the genes function as developmental regulators is the heterochronic pathway in *C. elegans* (Ambros and Horvitz, 1984). Genes involved in the heterochronic pathway function in a cascade, controlling and coordinating the timing of developmental decisions as the worm develops from initial larval stages to an adult. Furthermore, the high conservation of many genes in the pathway between *C. elegans* and other organisms signifies the importance of these factors (Grosshans et al., 2005; Lin et al., 2007; Moss and Tang 2003; Kanamoto et al., 2006 ; O'Farrell et al., 2008; Roush and Slack 2008; Pasquinelli et al., 2000). Thus, analyzing the heterochronic pathway in a morphologically simple organism like *C. elegans* has allowed us to gain insights into how a complex network of genes regulate the developmental decisions and can be used as a template to understand developmental gene regulation in higher organisms such as humans (Ambros and Horvitz, 1984; Reinhart et al., 2000; Rougvie 2001; Slack et al, 2000). Although this pathway has been investigated for several years, the relatively recent discoveries of the complexities of gene regulation such as non-coding RNA mediated control offer new opportunities to investigate the heterochronic pathway (Reinhart et al., 2000; Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros 2001; Miska 2005). Therefore, this thesis attempts to address the significance of miRNA-mediated as well as other control mechanisms in the heterochronic pathway in *C. elegans*.

Complex regulation of the heterochronic pathway in *C. elegans*

In this thesis, I have investigated the regulation by a subset of heterochronic genes that critically function in the *C. elegans* L4 molt, controlling the transition from the larval stage 4 to adulthood (Rougvie 2001). This transition is considered to be triggered by up-regulation of a miRNA *let-7,* which controls several down-stream targets to activate the adult specific differentiation program (Reinhart et al., 2000). The insights into the functioning of the genes in the heterochronic pathway have largely come from characterization of loss-of-function mutants (Reinhart et al., 2000; Rougvie 2001; Slack et al, 2000). Based on these studies a straightforward model can be derived to propose the mechanism of *let-7*-mediated control of this transition (Fig. 23), involving the exit from cell proliferation and resulting in the onset of differentiation in the hypodermal seam cell (Abrahante et al., 2003; Reinhart et al., 2000; Slack et al., 2000; Lin et al., 2003; Rougvie 2001).

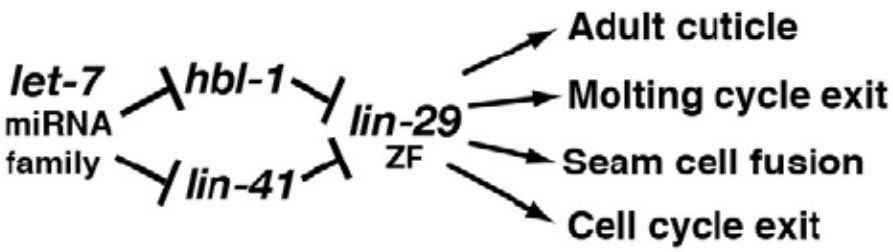


Figure 23. Model of how *let-7* and its family members regulate larval-to adult transition.
Major effectors involved in regulating various aspects of the larval-to-adult transition according to current understanding of the heterochronic pathway. (Harris and Horvitz 2011)

let-7's precocious expression was found to be sufficient to trigger terminal differentiation in the seam cells, when expressed under *lin-4* promoter two stages earlier. However, its expression resulted in production of alae and expression of *col-19::gfp* only at the L3-to-L4 molt and not earlier (Hayes and Ruvkun 2006). It is thus likely that additional factors may be required, which are not present during the larval stage 2 to trigger differentiation in the seam cells. Alternatively factors present during early larval stages may prevent *let-7's* function. One caveat in this study was the use of extra-chromosomal array, which limits the interpretation of the data presented in this study (Hayes and Ruvkun 2006). On the other hand the down-regulation of its direct target *lin-41* does not restore all the functions in the *let-7* mutant animals. Knockdown of *lin-41* when tested in *lin-41(ma104)* hypomorph as well as its canonical null allele *lin-41(n2914)* resulted in worms exhibiting 50% penetrance of partial precocious alae formation (Slack et al., 2000). This observation suggests that *let-7* likely regulates additional targets, which is not surprising considering the functional properties of the miRNAs in general that implicate them in regulation of multiple target transcripts to carry out their function.

One intriguing possibility was that *let-7* would directly target any of the core cell cycle regulators in order to mediate regulation of seam cell proliferation and differentiation. In support of this, previous studies involving microarray analyses have revealed several genes that regulate the cell cycle and cell proliferation to be responsive to alteration of *let-7* levels (Johnson et al. 2007). Additionally *let-7*b was suggested to target CDC25A in human cell culture, although it has remained unclear whether this interaction is physiologically relevant (Johnson et al., 2007; Huang et al., 2007). Therefore, we tested 44

genes involved in cell cycle regulation that could be potential *let-7* targets by screening for suppression of the bursting phenotype induced by *let-7* loss-of-function. Although suppression of the bursting phenotype was sufficient for the initial screening to assess direct *let-7* targets, detailed analysis now reveals that this screening strategy may not be without limitations, especially because of the observed vulvaless phenotype that may be at least partially responsible for the suppression of the bursting in *let-7* mutant worms. Additionally by using this screening method to select potential *let-7* targets, it is possible to miss the candidates whose knockdown resulted in suppression of hypodermal phenotype such as overproliferation and lack of differentiation in seam cells, which is observed in *let-7* loss-of-function mutants. In spite of these potential shortcomings, the screen did reveal two potential candidates *cdk-1* and *cdc-25.2* (Kim et al., 2010; Ashcroft et al., 1998; Ashcroft et al.,1999; Bashir and Pagano 2005; Boxem et al., 1999; Enserink and Kolodner 2010) whose depletion not only resulted in inhibition of cell division, but also partial rescue of alae formation, i.e. differentiation. This observation supported two hypotheses – (1) the existence of a crosstalk mechanism between cell cycle regulation and seam cell differentiation, and (2) the possibility of *cdk-1* and *cdc-25.2* being direct targets of *let-7*.

Although my data demonstrates that both *cdk-1* and *cdc-25.2* are not likely to be direct targets of *let-7,* we cannot not rule out that *cdk-1* functions further down-stream of *let-7*. This is because the CDK-1::GFP functional fusion protein (Shirayama et al., 2012) continues to be expressed in *let-7* mutant background during the adult stage. LIN-29 is a Cys2-His2 zinc-finger transcription factor, which is presently recognized as the most downstream regulatory gene in the heterochronic pathway (Rougvie 2001), regulating all four aspects of terminal hypodermal differentiation- i.e. adult cuticle formation, exit from molting, exit from cell cycle and seam cell fusion albeit at correct stage (Harris and Horvitz 2011). We therefore decided to assess the effect on *cdk-1* expression upon *lin-29* knockdown. Indeed, RNAi-mediated knockdown of *lin-29* resulted in elevated levels of CDK-1/GFP in the seam cells, similar to the effect of *let-7(n2853).* However, the mechanism of *lin-29* mediated negative regulation of CDK/GFP expression in seam cells remains unclear. Two possibilities can be hypothesized: (1) LIN-29 being a transcription factor negatively regulates *cdk-1* on the transcriptional level by directly binding to its promoter, and (2) alternatively LIN-29 functions through some intermediate factors, which regulates *cdk-1* expression. In order to assess the direct regulation of *cdk-1* by *lin-29*, we could perform chromatin immunoprecipitation experiment (ChIP) (Lee et al., 2006) to assess the LIN-29 protein binding to the promoter of *cdk-1*.

However the question remains of how down-regulation of the core cell cycle genes could trigger seam cell differentiation? A recent report demonstrates

that Cdk1 collaborates with Oct4 to inhibit mouse ES cells differentiation. This study demonstrated a direct interaction between Cdk1 and Oct4 and this interplay results in the inhibition of ES cell differentiation into trophectoderm (Lei et al., 2012). Based on this observation one could speculate that CDK-1 might interact with a similar pro-proliferative and anti-differentiation factor to inhibit differentiation and knocking down CDK-1 results in removal of such a block. Interestingly, *lin-29*, which is a downstream most gene in the heterochronic pathway is necessary to activate the adult-specific program, thus triggering differentiation of seam cells and is required for alae formation. One could speculate that knocking down *cdk-1* could release the block over *lin-29* resulting in alae formation in the *let-7* mutant background. However, the exact mechanism of this cross talk remains to be explored.

Although numerous papers and review articles have attempted to analyze all the heterochronic phenotypes observed in the seam cells, trying to place these genes in the pathway is not trivial considering multiple genes that work in parallel and partially redundantly, in the pathway regulating individual aspects of terminal differentiation in seam cells, ranging from cell cycle exit, to exit from molting, to seam cell fusion and alae formation (Rougvie 2001; Ambros and Horvitz, 1984; Ambros 2011; Liu et al., 1995). For instance (Lin et al., 2003) suggested that *let-7* targets *lin-41* and *hbl-1,* which act partially redundantly in the same, or parallel, pathways to affect the L/A switch by acting on *lin-29.* Oddly, although *lin-29* null mutants continually reiterate the larval cell division pattern (Bettinger et al., 1996; Rougvie 2001), precocious expression of LIN-29 resulting from loss of *lin-41* or *hbl-1* activity is insufficient to drive cell cycle exit and terminal differentiation in 100 % worms. Furthermore, another interesting observation made by (Lin et al., 2003) and (Vadla et al., 2012) is that unlike *lin-41* knockdown, down-regulating *hbl-1* does not result in suppression of the extra divisions of the seam cell nucleus. This observation does raise the prospect of *lin-41* regulating additional genes. Moreover, the present model does not clearly explain why *let-7* would regulate two different factors, whose output would eventually merge on a single transcription factor, which finally triggers the onset of terminal hypodermal differentiation.

Finally, to make the matters more complicated, the *let-7(n2853)* mutation results in only one additional seam cell division at permissive temperature, thus implying that the *let-7* mutations simply delay LIN-29 accumulation and terminal differentiation by one stage (Rougvie 2001). A major issue in performing the counting of the seam cells in adult stage of the *let-7* null worms impossible is the bursting of the worms. One possible way to overcome this issue is by performing RNAi against genes that result in the vulvaless phenotype without affecting the hypodermal phenotype observed in the *let-7* mutant worms. In contrast *lin-29* null mutants continually reiterate the larval

cell division pattern (Bettinger et al., 1996; Rougvie 2001), which may suggest the existence of another gene that acts together with, or in parallel to, *let-7* to promote activation of *lin-29.*

An insight into how LIN-41 protein may function

LIN-41 is involved in regulating critical developmental aspects in *C. elegans* and has been shown as the key target of *let-7* miRNA (Slack et al., 2000). Loss of regulation by *let-7* over *lin-41* has been implicated in mutant phenotypes in somatic tissues ranging from seam cell proliferation and differentiation defects to severe lethal phenotypes by bursting through the vulva. Recently Ecsedi and colleagues showed that *lin-41* alone is responsible for the bursting phenotype, since loss of regulation by *let-7* over *lin-41* but not any other *let-7* targets resulted in bursting of the worms (Ecsedi et al., 2015). In addition loss-of-function mutants of *lin-41* also result in defects in the germline that render the worms sterile (Tocchini et al., 2014; Spike et al., 2014; Slack et al., 2000). Although it is well established that loss-of-function of LIN-41 is responsible for these mutant phenotypes in multiple tissues, there is a limited mechanistic insight into how LIN-41 functions to regulate its targets. Furthermore, the reports on its mode of action are inconsistent resulting in many questions that remain to be answered (Rybak et al., 2009; Loedige et al., 2012, Chang et al., 2012).

LIN-41 binds to its target mRNAs and mediates post-transcriptional regulation of gene expression

LIN-41 belongs to the TRIM-NHL family of proteins and thus likely functions as a post-transcriptional regulator of gene expression (Slack et al., 2000). The preliminary indication supporting this premise was based on the observation that LIN-41 prevents the accumulation of the LIN-29 protein until the L4 molt (Slack et al., 2000), despite *lin-29* mRNA transcript being observed during larval stage 2 onwards (Rougvie and Ambros 1995; Bettinger et al., 1996).

However, analyzing the mechanism through which LIN-41 may regulate LIN-29 expression was complicated by the fact that the LIN-41 protein contains multiple domains that are implicated in different functions ranging from protein binding to ubiquitylation, in addition to its potential to bind to mRNAs (Slack et al., 2000; Rybak et al., 2009; Loedige et al., 2012, Chang et al., 2012). Nonetheless, two most likely mechanisms through which LIN-41 regulates LIN-29 expression were proposed. The first involved protein degradation via E3 ubiquitin ligase activity conferred by the RING domain. The second likely mechanism was through LIN-41 protein binding to the *lin-29* mRNA resulting

in translational inhibition (Slack et al., 2000).

A recent report suggested that LIN-41's ortholog, TRIM71 directly binds to the target mRNAs via NHL domain (Loedige et al. 2012). In the germline LIN-41 was suggested to regulate oocyte M-Phase entry through 3'UTR-mediated translational repression of *cdc-25.3* (Spike et al., 2014). Moreover, several isolated mutations in LIN-41 protein were mapped to the NHL domain and not the RING domain, which raised the question about the importance of RING domain's function in protein ubiquitylation-based mechanism for mediating LIN-29 regulation. Thus, although implicated in E3 ubiquitin ligase activity in *in vitro* studies (Rybak et al., 2009; Loedige et al., 2012, Chang et al., 2012), the RING domain needed for ubiquitin ligase activity appeared to be dispensable for mRNA repression (Loedige et al. 2012; Worringer et al. 2014). Additionally, Tocchini and colleagues demonstrated that despite mutating the key residues in the RING domain, this mutant form of LIN-41 appeared fully functional, thus arguing against LIN-41's E3 ubiquitin ligase activity (Tocchini et al., 2014). Collectively these observations supported the second mechanism, wherein LIN-41 was involved in the translational inhibition of LIN-29.

Our results demonstrated that LIN-41 protein does bind to *lin-29* mRNA, suggesting the possibility that LIN-41 regulates LIN-29 at post-transcriptional level by targeting *lin-29* mRNA and not its protein. In addition ribosome-profiling analysis provided further evidence in support of the LIN-41-mediated regulation occurring at translational level and does not seem to mediate *lin-29* mRNA degradation. Strikingly the ribosome-profiling analysis also revealed additional potential mRNA targets of LIN-41 such as *mab-10, dmd-3* and *mab-3* (F. Aeschimann, unpublished data), which were further validated by performing LIN-41 IP experiments. Interestingly *mab-3* and *dmd-3* are transcription factors, which were thought to be indirect targets of LIN-41 in male tale-tip development (Del Rio-Albrechtsen et al., 2006; Mason et al., 2008). MAB-10 is a cofactor that associates with LIN-29 in *C. elegans,* which regulates terminal differentiation and the transition from larva-to-adult stage in *C. elegans* (Harris and Horvitz 2011). Strikingly the NAB-interaction domain of LIN-29 is conserved in Kruppel-family early growth response (EGR) proteins. In mammals, EGR proteins control the differentiation of multiple cell lineages, and EGR1 acts with NAB proteins to regulate luteinizing hormone β subunit (Harris and Horvitz 2011). Furthermore, a recent report from Worringer and colleagues identified EGR1 as a major target of LIN41 at the mRNA level, down-regulation of which is required for induced pluripotent stem cell generation (Worringer et al., 2014). Collectively these observations demonstrate that the factors as well as their interactions, which mediate developmental regulation is conserved between worms and humans.

The LIN-41 protein target binding region within *lin-29* mRNA

Several reports suggest that LIN-41 binds to the 3'UTR region of its targets. For example, TRIM71 binds to the 3'UTR of its target mRNAs to mediate post-transcriptional regulation (Chang et al., 2012; Loedige et al., 2012). Another example was of the direct interaction of the BRAT protein with the *hunchback (hb)* mRNA through a 100-nucleotide long region in the 3'UTR of *hb* containing two Nanos response elements. Binding of BRAT to the 3'UTR region of *hb* results in translational inhibition by interacting with the cap-binding protein d4EHP (Cho et al., 2006). A recent report suggested LIN-41's mRNA binding potential to the 3'UTR of *cdc-25.3* (Spike et al., 2014). Surprisingly in all the previous reported cases LIN-41 targeted 3'UTR regions to mediate regulation. By contrast, our results demonstrated that LIN-41 mediates *lin-29* regulation by binding to the 5'UTR region in the *lin-29* mRNA, and not the 3'UTR region. These novel findings raise multiple questions such as how does LIN-41 recognize the binding site on *lin-29* mRNA? Does the recognition involve a specific sequence or does LIN-41 recognize secondary structure in the 5'UTR region. Furthermore, how does LIN-41 protein binding to the 5'UTR region of *lin-29* inhibit its translation? Does LIN-41 recruit additional proteins that are involved in mediating translation repression?
A well-characterized example of RNA binding protein-mediated regulation through the 5'UTR region is of iron regulatory protein (IRP1 and 2). These proteins recognize a conserved stem loop structure known as the iron response elements (IRE). If we were to extend this model to the LIN-41 mediated translation inhibition, we could speculate that the LIN-41's NHL domain might recognize a secondary structure in the 5'UTR region of *lin-29* mRNA, causing a steric inhibition of the binding of 40S ribosomal subunits to the transcript, affecting its recruitment. Alternatively one could also envision that LIN-41 could block ribosomal scanning thus preventing the translation of *lin-29* mRNA. However, the exact mechanism of how LIN-41 mediates translational inhibition of *lin-29* remains to be established.

Does LIN-41 bind to any protein partners?

Being a member of the TRIM-NHL family of proteins, it has been suggested that LIN-41 could potentially bind to proteins due to the presence of the domains such as RING, B-Box, Coiled-Coil and NHL, there are only two studies where they found proteins being Co-IPed with LIN-41. Analysis carried out in mouse ES cells demonstrated that mLin-41 binds to the Argonaut proteins as well as Dicer upon LIN-41 Co-IP, albeit in RNA dependent manner (Rybak et al., 2009; Chang et al., 2012). Later Chen and colleagues showed that in the neural progenitor cells, mLin-41 does not bind to AGO2 but instead binds to SHCBP1, which is an important component of FGF signaling in

neural progenitor cells (Chen et al., 2012). In addition other TRIM-NHL family proteins have been implicated in direct protein-protein interaction (Wulczyn et al., 2011). Collectively these reports did put forth an intriguing possibility of LIN-41's potential to interact with other proteins.

In the context of our finding that LIN-41 binds to the 5'UTR of *lin-29,* mediating translational repression, we wanted to further investigate if we could uncover potential LIN-41 protein binding partners that could be involved in functioning together with LIN-41 in mediating translational repression of LIN-29. However, we encountered multiple challenges during our attempts to assess potential LIN-41 protein binding partners, ranging from the levels of expression of LIN-41 to problems eluting out LIN-41 efficiently, which typically reflects denaturation on the beads. In future studies we envision using a functional truncated version of LIN-41 that might solve the denaturation issue that we faced. Furthermore, using a smaller tag with the inclusion of TEV site while constructing the *lin-41* transgene may improve the pull down of LIN-41 as well as the elution of the protein during immunoprecipitation. Furthermore, recent advances in the mass spectrometry methods may increase the sensitivity by detecting weak signals from potential LIN-41 protein partners.

Despite these issues, Co-IP of LIN-41 resulted in pull-down of multiple proteins that are implicated in RNA metabolism. In mixed stage IP where LIN-41 protein originates predominantly from the adult germline, an interesting hit was the ATP dependent dead-box helicase CGH-1. *cgh-1* is involved in translational regulation of mRNAs in the germline (Rajyaguru and Parker, 2009; Boag et al., 2008; Navarro et al., 2001). Interestingly immunofluorescence analyses performed by Loedige and colleagues revealed LIN-41's partial co-localization with DDX6, which is a homolog of CGH-1 (Loedige et al., 2012). Strikingly, we also found another dead box helicase, LAF-1 (Hubert and Anderson 2009), associating with LIN-41 upon analyzing the IP eluate obtained from larval stage 3 worm lysate. Nonetheless, there is a distinct possibility that these dead box helicases are getting pulled down due to indirect binding with LIN-41, mediated by RNA, since they are *bona-fide* components of various RNA granules and known to associate with multiple RNAs (Minshall et al., 2009). However, considering the fact that these dead box helicase are involved in various aspects of mRNA regulation such as translational regulation, storage and degradation (Rocak and Linder 2004; Linder and Janowsky 2011; Gustafson and Wessel 2010), further investigation is needed in order to assess if any of these factors are genuine interacting partners of LIN-41 or would play a functional role in LIN-41 mediated regulation of gene expression. Furthermore, LIN-41/TRIM71 proteins are known to be associated with P bodies, which are implicated in mRNA storage and/or degradation (Rybak et al., 2009; Chang et al., 2012). Based on the pull down of these dead box helicases, one could also

speculate that LIN-41 functions by binding to *lin-29* mRNA and sequestering it in an appropriate compartment of the cell, thus preventing its translation until needed with the assistance from proteins in P-bodies (Rybak et al., 2009; Sheth and Parker 2003; Parker and Sheth 2007).

In addition to binding RNA, TRIM-NHL proteins have been shown to modulate functions of other proteins, for example TRIM3 appears to regulate p21 (Liu et al., 2014). One could imagine LIN-41 mediated sequestering of its target proteins thus making them unavailable until needed. Previously, TRIM2 and TRIM3 were reported to interact with the globular domain of MYOSIN Va and TRIM3 was shown to influence endosome recycling (El-Husseini and Vincent, 1999; Ohkawa et al., 2001; Yan et al., 2005). Furthermore, Trim32 was also shown to physically interact with the head and neck region of the myosin heavy chain, which was mediated by the coiled-coil domain (Frosk et al., 2002; Saccone et al., 2008; Kudryashova et al., 2005). Structural similarity of the TRIM-NHL proteins might suggest that these functions could be shared among family members and that LIN41 might also interact with MYOSIN. Strikingly we found multiple myosin proteins being coimmunoprecipitated with LIN-41. Such interactions could provide a potential clue about how LIN-41 may function in regulating translation inhibition (Piekny et al., 2003). One could speculate LIN-41 binding to its target mRNAs in order to transport them with the help of myosin based molecular motors. Additionally, data from Ecsedi and colleagues have demonstrated that LIN-41 plays a critical role in vulval morphogenesis (Ecsedi et al., 2015). Our IP data could provide an alternative hypothesis where LIN-41 plays a structural role not unlike the LIN-41 homolog *wech* in Drosophila melanogaster*,* which has been implicated in establishment of muscle attachment to the body wall (Loer et al., 2008).

Previous studies conducted in *in vitro* cell culture by Rybak et al (2009) and Chang et al (2012) reported mLin41 binding to AGO proteins as well as DICER. However, immunoprecipitating our tagged LIN-4, we did not find *alg-1* or *alg-2*, which are homologs of AGO proteins nor DCR-1 protein associating with LIN-41. It is possible that *C. elegans* LIN-41 does not bind to these proteins or we could not detect these proteins due to their low abundance in the Co-IP eluate.

Although the LIN-41 Co-IP analysis resulted in a potentially interesting list of putative interacting partners of LIN-41, one significant question was, whether any of the proteins that we observed in LIN-41 Co-IP were involved in functioning with LIN-41 to regulate post-transcriptional regulation of gene expression. Our attempt to validate the putative LIN-41 protein-binding partners using *lin-29* reporter as a sensor did not yield further supporting data. However, there are a few caveats in this screening method that could not be addressed in this study. The readout of the reporter had to be assessed in a

narrow time window during larval stage 3, since the reporter expression starts getting up-regulated from larval stage 4 and beyond as the LIN-41 mediated repression is lost. This complicated the analysis due to the possibility that the depletion of the IP hits was inefficient during early stages larval stages, since the reporter worms were added to the RNAi plates from L1 stage onwards. Furthermore, what needs to be addressed is the efficiency of the knock down of these IP hits by RNAi by assessing mRNA levels as well as the protein levels. Another possibility is that the putative LIN-41 binding partners could be tissue specific, hence did not result change in the levels of the *lin-29* reporter in the hypodermis.

One possible way to circumvent this problem, which might reveal directly the potential protein binding partners that are important in the context of translational regulation of *lin-29* mRNA by LIN-41 is to perform biotinylated RNA pull down of *lin-29.* This might reveal the possible proteins that associate with *lin-29* mRNA as well as LIN-41 protein, which could be involved in its translational inhibition.

Nonetheless, our LIN-41 Co-IP data has provided a potential list of protein interacting partners of LIN-41, which need further validation for assessing genuine proteins partners that associate with LIN-41.

LIN-41 domain function analysis

lin-41 mutants available from two studies (Tocchini et al. 2014 and Spike et al. 2014) allowed us to gain further insight into somatic phenotypes arising due to mutations in individual domains of LIN-41.

Slack and colleagues suggested that *lin-41* is unlikely to be the sole input to *lin-29* regulation. The precocious phenotype measured by counting the alae formation in L3 molt in *lin-41(n2914)* canonical null mutant is only 50% penetrant, since not all worms exhibit alae at L3 molt, also in addition to not all seam cells expressing alae (Slack et al., 2000). By contrast, upon counting the worms expressing precocious alae due to loss-of-function of LIN-41 resulting from premature stop codon in the RING and the B-Box, we observed >80% of these mutant worms displaying partial alae at L3 molt. This observation raises an interesting question, why a canonical null allele of *lin-41* would show lower penetrance as compared to the other null alleles that we assessed. One possibility is of residual expression of LIN-41 due to read through the stop codon. Alternative explanation would be an issue with counting the worms expressing precocious alae. When the balanced *lin-41(n2914)* animals segregate null worms, both the balanced and null worms look wild type at L3 molt. Both *lin-41(tn1505)* and *lin-41(tn1491)* contained a

gfp tagged balancer, which enabled us to count only the *lin-41* null worms that did not contain the balancer. Furthermore, the premature stop codon in the coiled-coil domain resulted in only 50 % worms displaying alae in L3 molt. This decrease in the number of worms displaying precocious alae due to the premature stop codon in coiled-coil domain does suggest the possibility of expression of either the truncated version of the protein or read through the stop codon resulting in residual expression of LIN-41.

Interestingly both the missense mutations in the NHL domain resulted in a very low penetrance of precocious alae formation at L3 molt. This observation suggested that the NHL domain is either partially functional in these mutants or dispensable, in which case it may indicate additional domains of LIN-41 functioning with NHL to regulate the downstream targets. Indeed as reported by Loedige and colleagues, NHL domain was important for targeting and mRNA binding, whereas the coiled-coil and filamin domains were shown to be required for mRNA regulation (Loedige et al., 2012). Strikingly, the NHL domain mutants resulted in moderate to strong up-regulation of the *lin-29* reporter in 100% of the worms. These observations did raise a question about the discrepancy between our *lin-29* reporter expression and precocious alae formation. Although we observed 100% worms expressing precocious *lin-29* reporter, we did not see 100 % worms expressing alae. As mentioned before, *lin-29* encodes a zinc-finger transcription factor and is considered as the most downstream regulatory gene identified in the heterochronic gene pathway (Bettinger et al., 1996; Rougvie 2001), which regulates all four aspects of terminal hypodermal differentiation (Harris and Horvitz 2011). Moreover, all the *lin-41* mutants resulted in partial alae formation rather than complete alae. One possible reason to which we could attribute this observation is that our *lin-29* reporter fails to capture all regulatory elements. Additionally, the *lin-29* gene encodes two isoforms, which could possibly explain this discrepancy, if only one of the isoforms is regulated and the others are not. Alternatively, alae formation might require regulation of another factor working in parallel to *lin-29*, which is expressed during larval-to-adult transition.

Analysis of the LIN-41 mutant worm lines obtained from (Tocchini et al., 2014) revealed that mutating RING and B-Box domains individually did not result in any somatic defects. However, mutating a single residue in the NHL domain resulted in observable somatic defects. Furthermore, the NHL domain mutant resulted in precocious expression of the *lin-29* reporter whereas the RING domain mutant did not, thus suggesting that the RING domain is dispensable for *lin-29* regulation. This observation further highlights the role of NHL domain in mRNA binding and regulation of *lin-29* through translational inhibition.

Overall the LIN-41 domain mutant analysis suggests that the NHL domain is critical for the somatic function of the protein. The most likely mechanism through which the NHL domain may function is via mRNA binding, thus regulating gene expression, as observed in our *lin-29* reporter expression. However, the possibility of other mechanisms cannot be ruled out and further investigation into the function of remaining domains in required.

8. Outlook

To sum up, the work described in this thesis contributes to better understanding of how the heterochronic genes function in regulating larval-to-adult transition in *C. elegans* worm. We conclude that *let-7* regulates *cdk-1* expression indirectly through *lin-29* transcription factor (Fig. 24). Furthermore, our work has finally addressed how LIN-41 regulates its down-stream target *lin-29.* We demonstrated LIN-41's ability to bind to *lin-29* mRNA in the 5'UTR region, thus regulating its protein levels by inhibition of translation. Furthermore, the LIN-41 immunoprecipitation experiment uncovered additional direct mRNA targets of LIN-41 as well as list of putative protein interaction partners. Future work would involve how LIN-41 regulates these additional mRNA targets as well as validation of the protein-binding partners of LIN-41.

It would be interesting to re-assess the function of *lin-29* in depth and how this transcription factor may regulate its down stream targets in *C. elegans* in order to carry out the larval-to-adult transition. Evidence from previous studies has suggested that multiple inputs may eventually converge on *lin-29* (Rougvie 2001; Harris and Horvitz 2011), since it is involved in regulating all aspects of seam cell differentiation. Moreover, in recent study EGR1 was identified as a major target of LIN-41 at the mRNA level (Worringer et al., 2014). The transcription factor EGR1 contains a conserved LIN-29 domain and interacts with the cofactors NAB1 and NAB2, which are homologues of the LIN-29 cofactor MAB-10 in *C. elegans* (Harris and Horvitz 2011). Additionally EGR1 is an important activator of genes expressed in differentiated fibroblasts that needs to be repressed during iPSC induction (Fragola et al., 2013), which collectively point in the direction of *lin-29* playing a critical role in promoting cell cycle exit as well as differentiation.

Finally why the knock down of *cdk-1* or *cdc-25.2* result in alae formation i.e. differentiation needs to be addressed and the mechanism behind this cross talk needs investigation.

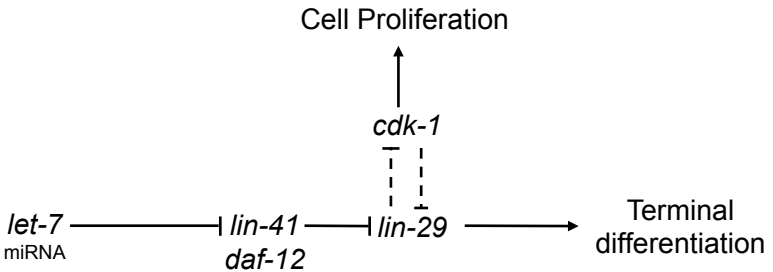


Figure 24. Model of heterochronic genes regulating hypodermal seam cell proliferation and differentiation.

9. Materials and Methods

Project I

Strains

Strains were maintained using standard procedure on OP50 bacteria seeded on Nematode growth media (NGM) plates (Stiernagle, 2006). Bristol N2 strain was used as wild type. Strains carrying temperature sensitive allele of *let-7(n2853ts)* were maintained at the permissive temperature of 15 °C and shifted to 20 °C and 25°C to carry out the experiments. For the experiments synchronous worm population were obtained by collecting embryos from bleached adults and synchronizing larvae by starvation in M9 on rotating wheel before feeding. All the transgenic reporter lines were generated using Mos1-mediated Single-Copy transgene Insertion technique (Frokjaer-Jensen et al., 2012; Frokjaer-Jensen et al., 2008). The CDK-1::GFP cb-unc-119(+) (II) unc-119 (III) expressing a functional fusion protein from the native *cdk-1* promoter was obtained from Dr. Masaki Shirayama (Shirayama et al.,2012). All the strains used in this study are listed in (Table 3)

RNAi screen for *let-7* mutant phenotype suppression

Based on the annotations for known or predicted cell cycle regulators in the *C. elegans* Wormbase, 44 genes were tested by performing RNAi screen using the RNAi clones from Ahringer library (Kamath and Ahringer 2003). The worms were fed with HT115 bacteria expressing dsRNA (Timmons et al., 2001). As negative control worms were fed with an insertless plasmid (L4440) (Timmons and Fire, 1998). Both *let-7(n2853)* and *let-7(mn112)* worms grown under RNAi conditions were scored for the bursting phenotype suppression as well as reversal of over proliferation and differentiation defect observed in *let-7* mutant in the seam cells. For detailed analysis of the restoration of *col-19* expression, *col-19::gfp; let-7(n2853)* worms were added on suppressor RNAi plates and tested for *col-19* expression after 48 hours at 25°C. Worms were observed under Zeiss Z-1 microscope, running AxioVision SE64 software.

Generating reporters using single-copy transgene insertion

The hypodermal-specific *wrt-2* promoter (Aspöck et al.,1999) and 3'UTRs of *cdk-1* and *cdc-25.2* were amplified using the listed primers (Table. 2) from N2 worm genomic DNA. DNA fragments were inserted into the appropriate Gateway donor vectors. *Pwrt-2*, gfp::h2b::PEST (pBMF2.7) and individual

3’UTR entry vectors were recombined into the MosSCI-compatible pCFJ150 plasmid for chromosome II using Multisite Gateway Technology (Life Technologies, Carlsbad, CA, USA) according to the supplier’s protocol. All plasmids were verified by sequencing. Using Mos1-mediated Single-Copy transgene Insertion (MosSCI) the transgenes were integrated at defined genomic locus in chromosome II (Frokjaer-Jensen et al., 2012; Frokjaer-Jensen et al., 2008). Before back crossing the transgenic lines, insertion of transgenes was verified by PCR, following which the lines were backcrossed three times.

Microscopy

DIC and fluorescent images were obtained using Zeiss Z-1 microscope and AxioVision SE64 (release 4.8) software (Carl Zeiss, Oberkochen, Germany).

Reporter regulation

To assess regulation of *cdk-1* and *cdc-25.2* 3’ UTR reporter transgenes by *let-7*, synchronized worms were grown on NGM plates seeded with OP50 bacteria for 36 hours at 25°C on plates. Worms were observed on a Zeiss Z-1 microscope with Axiovision software using Nomarski DIC and fluorescence microscopy.

Project II:

Strains

Strains were maintained using standard procedure on OP50 bacteria seeded NGM plates (Stiernagle, 2006). Bristol N2 strain was used as wild type.
For the experiments involving synchronous worm population were performed by collecting embryos from bleached adults and synchronizing larvae by starvation in M9 on rotating wheel before feeding. All the transgenic reporter lines were generated using Mos1-mediated Single-Copy transgene Insertion technique (Frokjaer-Jensen et al., 2012; Frokjaer-Jensen et al., 2008).
All the strains used in the study are listed in (Table. 3)

Cloning

Cloning was performed by Phusion Hot Start II High-Fidelity DNA Polymerase, (catalog number: F-549L, Thermo Scientific) according to the supplier's protocol using listed primers (Table. 2).

Generating functional tagged *lin-41* transgenic strain using single-copy transgene insertion

The *lin-41* promoter, ORF and its 3'UTR were PCR amplified using the listed primers from N2 worm genomic DNA. The amplified DNA fragments were inserted into the appropriate Gateway donor vectors. *Plin-41*, 1x*flag-gfp-lin-41* (ORF) and *lin-41* 3'UTR entry vectors were recombined into the MosSCI-compatible pCFJ150 plasmid for chromosome II using Multisite Gateway Technology (Life Technologies, Carlsbad, CA, USA) according to the supplier's protocol. All plasmids were verified by sequencing. Using Mos1-mediated Single-Copy transgene Insertion (MosSCI) the transgenes were integrated at defined genomic locus in chromosome II (Frokjaer-Jensen et al., 2012; Frokjaer-Jensen et al., 2008). Before back crossing the transgenic lines, insertion of transgenes was verified by PCR, following which the lines were backcrossed three times.

Generating reporter worm lines for assessing LIN-41 mediated LIN-29 regulation

lin-29 promoter and the 3'UTR were PCR amplified using listed primers from N2 worm genomic DNA. The amplified DNA fragments were inserted into the appropriate Gateway donor vectors. Reporter constructs were generated by

recombining following entry vectors *Pdpy-30, gfp::h2b::pest (pBMF2.7), lin-29* 3'UTR, *Plin-29, pCM5.37* into the MosSCI-compatible pCFJ150 plasmid for chromosome II using Multisite Gateway Technology (Life Technologies, Carlsbad, CA, USA) according to the supplier's protocol. A second reporter containing *Plin-29* and *lin-29* 3'UTR, however containing pCM1.151 plasmid for *mCherry* expression was generated by recombining following entry vectors into the MosSCI-compatible pCFJ210 plasmid for chromosome I using Multisite Gateway Technology (Life Technologies, Carlsbad, CA, USA) according to the supplier's protocol.

Microscopy

DIC and fluorescent images were obtained using Zeiss Z-1 microscope and AxioVision SE64 (release 4.8) software (Carl Zeiss, Oberkochen, Germany).

Antibodies and Western blotting

Polyclonal affinity-purified (ELISA) rabbit anti-LIN-41 antibody was generated by SDIX in Newark, DE, USA. (4796) and created against the VKNLKLSVLISQAESLQSKQIDLQQAIQTATKLMDSSDCDEMVLRQVFEKLA SCQMGNEGTEPNNNILNVLMLACQVNEDDRLKFTAPQDGILLNKARQF sequence (residues 587–686).
For western blot analyses, lysates or immunoprecipitates (IPs) were boiled in Laemmli buffer, separated by SDS-PAGE and electro-transferred to PDVF membranes. The following primary antibodies were used: rabbit anti-LIN-41 antibody (4796)- 1:3000; mouse anti-GFP (Roche, Penzberg, Germany) 1:6000
Secondary antibody used: anti-rabbit/ anti-mouse horseradish peroxidase-conjugated secondary antibody (1:7500) (GE Healthcare, Little Chalfont, UK) reaction. The membranes were treated with ECL Western Blotting Detection Reagents,
and protein bands were detected using Amersham Hyperfilm ECL.

Immunoprecipitation

Both larval stage 3 worms and mixed stage worms were harvested in M9 and snap-froze in liquid nitrogen.
Mixed stage worms were lysed with a Dounce Tissue Grinder (BC Scientific, Miami, FL, USA) in lysis buffer containing 50 mM HEPES/KOH pH 7.4, 150 mM KCl, 5 mM MgCl2, 0.1% Triton X-100, 10% glycerol w/vol and protease inhibitors (Protease Inhibitor Cocktail Tablets, EDTA-free, Roche). Lysates

were cleared at 16,000xg for 20 min at +4 °C. In the case of larval stage 3 worms frozen pellets were added to either mortar and pistil or the ball mill disrupter and pulverized into fine powder to which 50 mM HEPES/KOH pH 7.4, 150 mM KCl, 5 mM MgCl2, 0.1% Triton X-100, 10% glycerol w/vol and protease inhibitors (Protease Inhibitor Cocktail Tablets, EDTA-free, Roche) was added. Lysates were cleared at 16,000xg for 20 min at +4°C. Protein concentration was determined using Bradford reagent. Lysates containing FLAG-GFP-LIN-41 and control FLAG-GFP-SART3 were incubated with Anti-FLAG M2 magnetic beads (Sigma-Aldrich- M8823-1ML) for 3 h. Washes were performed in lysis buffer. Elution was achieved by incubation with 1 mg/ml 3x FLAG peptide (Sigma-Aldrich). For RNA extractions, TRI Reagent (Molecular Research Center) was directly added to the magnetics beads.

RT-qPCR analysis

All steps were performed on ice. RNA concentrations were measured by nanodrop. 900 ng of input/unbound RNA were combined with 1 µl of random hexamers and RNase-free water was added to a total volume of 12 µl. 7.5 µl of IP RNA (50 % of the IP) were combined with 1 µl of random hexamers and 3.5 µl of RNase-free water. The reverse transcription master mix was prepared (per reaction: 4 µl ImProm-II 5x Reaction Buffer, 1.5 µl MgCl2 (25 mM), 1 µl dNTP mix (7.5 mM each), 0.5 µl RNasin (40 U/µl), 1 µl ImProm-II Reverse Transcriptase). To each RNA (12 µl), 8 µl of master mix were added and reverse transcription was performed in a PCR machine with the following program: 25 °C (5 min), 42 °C (60 min), 70 °C (15 min), 4 °C (hold). cDNA was diluted by a factor of 1:20, 10 µl of cDNA were added to 190 µl of nuclease-free water. A master mix was prepared for each primer pair by combining 10 µl 2x SYBRGreen PCR master mix (Applied Biosystems; 4309155), 0.5 µl of each primer (10 µM) (Table. 2) and 4 µl of nuclease-free water per reaction. On the PCR plate, 5 µl of cDNA were added to 15 µl of QPCR master mix for each reaction and the QPCR was performed on a StepOnePlus realtime PCR system (Applied Biosystems). For each condition, technical triplicates were measured and the average CT calculated.
For calculating fold enrichments identical lysate amounts were taken as input for all the IPs (3 mg of protein per IP) and identical RNA amounts were taken as input for the reverse transcription for the input samples (900 ng). Relative enrichment (RE) value in the IP for each measured mRNA was calculated with: RE = 2^(-ΔCT) = 2^(-(CT (IP) - CT (input))) = 2^(CT (input) - CT (IP)). “RE” values were calculated separately for the LIN-41 IP, the SART-3 IP and the N2 control IP. Enrichments were normalized by calculating them relative to the N2 “RE” value and plotted.

REnorm(LIN-41) = RE(LIN-41) / RE(N2)
REnorm(SART-3) = RE(SART-3) / RE(N2)

Mass-spectrometry

TCA precipitated and acetone washed protein pellets were dissolved in 0.5 M Tris, pH 8.6, 6 M guanidinium hydrochloride, reduced in 16 mM TCEP for 30 min, alkylated in 35 mM iodoacetamide for 30 min in the dark and and cleaved with LysC (0.2μg) for 6 hours. The proteins were digested at 37°C with trypsin (0.2μg) (Promega, Madison, USA). End vol. 80ul. 20ul of the samples were acidified with 1ul 20%TFA. The generated peptides were analyzed using LTQ Orbitrap Velos (Thermo Fisher Scientific) with Easy-nLC and 75umx2cm PepMap Trap (101411) and 75um x 25cm ReprosilPur C18 (04014007). Gradient: 0-5min 2-8%B in A, 5-55min 8-28%, 55-63min 28-36%, 63-67min 36-44%, 67-72min 44-80%, 72-77min 80%, 77-80min 80-2%, 88-90min 2%. Mascot (Matrix Science, London, UK) searching UniProt data base version 2013-11 was used to identify the peptides.

Table. 2

Primer	Purpose	Sequence (5'-3')
***let-7* project primers**		
pWRT2 GW F attB4	Gateway cloning wrt-2 promoter	GGGGACAACTTTGTATAGAAAAGTTGTATGACCATGATTACGCCAAG
pWRT2 GW R attB1r	Gateway cloning wrt-2 promoter	GGGGACTGCTTTTTTGTACAAACTTGCCCGAGAAACAATTGGCA
lin-41_3U F	Gateway cloning lin-41 3'UTR	GACACTTTCTTCTTGCTCTTTAC
lin-41_3U R	Gateway cloning lin- 41 3'UTR	GAAACTCGACTAGGAATTCGAG
cdc-25.2 GW F attB2r	Gateway cloning cdc-25.2 3'UTR	GGGGACAGCTTTCTTGTACAAAGTGGAATTATTCCTCCTTGATTTC
cdc-25.2 GW R attB3	Gateway cloning cdc-25.2 3'UTR	GGGGACAACTTTGTATAATAAAGTTGCTTTCGCCAAATCACATTAC
cdk-1 GW F attB2r	Gateway cloning cdk-1 3'UTR	GGGGACAGCTTTCTTGTACAAAGTGGTGATGTAATTCATTCATCATCA
cdk-1 GW R attB3	Gateway cloning cdk-1 3'UTR	GGGGACAACTTTGTATAATAAAGTTGTCTTAATTCCCTATTCTCATTTA
LIN-41 project primers		
plin-41 GW attB4 F	Gateway cloning *lin-41* promoter	GGGGACAGCTTTCTTGTACAAAGTGGTACCACGCAGACAAGGAGCTAC
plin-41 GW attB1r R	Gateway cloning *lin-41* promoter	GGGGACAACTTTGTATAATAAAGTTGTCACTTTTTCCAAGTCTGAAAAGG
lin-41 GW attB2r F	Gateway cloning *lin-41* 3'UTR	GACACTTTCTTCTTGCTCTTTAC
lin-41 GW attB3 R	Gateway cloning *lin-41* 3'UTR	GAAACTCGACTAGGAATTCGAG
C term lin-41 GW attB2 R	Gateway cloning *lin-41* C terminal tagging	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTA CTT GTC ATC ATC ATC TTT ATA
GFP F+ linker	lin-41 C terminal tagging GFP with linker sequence	GGTAGCGGCAGCGGTAGCATG AGT AAA GGA GAA GAA CTT TTC
C term lin-41 GW attB1 F	Gateway cloning *lin-41* C terminal tagging	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGCGACCATCGTGCCA
C term lin-41 R+linker	lin-41 Rev primer minus stop codon+ linker for C terminal tagging	GCTACCGCTGCCGCTACCGAAGACACGGATGCAATTGTT
lin-41+linker F	N terminal tagging Linker + lin-41	GGTAGCGGCAGCGGTAGCGCGACCATCGTGCCATG
N term lin-41 GW R attB2	Gateway cloning N terminal tagging lin-41 reverse primer with stop codon	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTA GAA GAC ACG GAT GCA ATT
N term flaggfp GW F attB1	Gateway cloning N terminal tagging lin-41 Flag GFP fwd primer with attB1 site	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGACTACAAAGACGATGACGA
GFP R+ linker	N terminal tagging GFP reverse primers with linker sequence	GCTACCGCTGCCGCTACCAGC TGG GTC TGA AAA TAC AGG TT
lin-41 N-tag seq1	N terminal sequencing primer for lin-41	TGGACTACAAAGACGATGACG
lin-41 N-tag seq2	N terminal sequencing primer for lin-41	TCCACACAATCTGCCCTTTC
lin-41 N-tag seq3	N terminal sequencing primer for lin-41	CACCACAGCAGCAACCACAG
lin-41 N-tag seq4	N terminal sequencing primer for lin-41	CGGAGGACACCAGCAACAATC
lin-41 N-tag seq5	N terminal sequencing primer for lin-41	GGATCTCAGCCACAACAACAAC
lin-41 N-tag seq6	N terminal sequencing primer for lin-41	GATCCGTTTCAAGATTCTCCAC
lin-41 N-tag seq7	N terminal sequencing primer for lin-41	TATTCACAAGCCAGTCGGAG
lin-41 N-tag seq8	N terminal sequencing primer for lin-41	GCAATGTGAGAAGACTGGTG
lin-41 N-tag seq9	N terminal sequencing primer for lin-41	TCAAATTCACAGCTCCACAGG
lin-41 N-tag seq10	N terminal sequencing primer for lin-41	CCCATTTGGAACAAGCTATGC
lin-41 N-tag seq11	N terminal sequencing primer for lin-41	ATCATCGTGTCCAGGTATTCG
lin-41 N-tag seq12	N terminal sequencing primer for lin-41	CAAATGCCACAAGAGCTACC
lin-41 C-tag seq13	C terminal sequencing primer for lin-41	TCCTTGTCATCAGCAGCCCTC
lin-29 attB2 F2	Gateway cloning lin-29 3'UTR region amplification	GGGGACAGCTTTCTTGTACAAAGTGG TAA TTT TAA TTT TTT TTT GAA TTT TTT CTA A
lin29 attB3 R2	Gateway cloning lin-29 3'UTR region amplification	GGGGACAACTTTGTATAATAAAGTTG ATA CAT AAT CGT TTA TAT TTT CAA TC
mab3 attB2 F	Gateway cloning Mab-3 3'UTR amplification	GGGGACAGCTTTCTTGTACAAAGTGG TAA GAT CTA TAA TTT TGA CCA ATT AT
mab3 attB3 R	Gateway cloning Mab-3 3'UTR amplification	GGGGACAACTTTGTATAATAAAGTTG CGT GGA GCA GAA CGT CTC
dmd-3 attB2 F	Gateway cloning dmd-3 3'UTR amplification	GGGGACAGCTTTCTTGTACAAAGTGG AAA CTC TAA AAT AGT TTG AAT TTT TAA ATT
dmd-3 attB3 R	Gateway cloning dmd-3 3'UTR amplification	GGGGACAACTTTGTATAATAAAGTTG CCC GAA GTG TCA GCC TAT ATT
mab10 attB2 F	Gateway cloning Mab-10 3'UTR amplification	GGGGACAGCTTTCTTGTACAAAGTGG ATC TTG AAG CTC GGA TTT CCA T
mab10 attB3 R	Gateway cloning Mab-10 3'UTR amplification	GGGGACAACTTTGTATAATAAAGTTG TGT TAC GGG AAT CAT GTC TTC
mCherry attB1 F	Gateway cloning mCherry H2b Pest amplification	GGGGACAAGTTTGTACAAAAAAGCAGGCTCC ATG GTC TCC CTT AGC CAT GGC TT
mCherry attB2 R	Gateway cloning mCherry H2b Pest amplification	GGGGACCACTTTGTACAAGAAAGCTGGGTC TTA CTT GCT GGA AGT GTA CTT GGT G
lin-29 promoter attB4 F	Gateway cloning lin-29 Promoter amplification	GGGG ACA ACT TTG TAT AGA AAA GTT GGG GAT ATA TTT TGA TCG CTA CTC AAC A
lin-29 promoter attB1 R	Gateway cloning lin-29 Promoter amplification	GGGG ACT GCT TTT TTG TAC AAA CTT GG TGC GTT GAA GAA GTT GGC TTG A
plin29 seq1	Primers for lin-29 promoter sequencing	ACAAGGTGGTGGTAAAAAGTGTCTGC
plin29 seq2	Primers for lin-29 promoter sequencing	TAATTTCTCTGCCACCTTCAATTTTATCAA
plin29 seq3	Primers for lin-29 promoter sequencing	GAAAAGAGCCTACTAAATATTGGAACT
plin29 seq4	Primers for lin-29 promoter sequencing	TTATTTCCGGCAAATCGGAGCATTGC
plin29 seq5	Primers for lin-29 promoter sequencing	CACAGCTGTGTGCACTGTGCACT
laf1 attB F	Primers for creating *laf-1* RNAi clone	GGGGACAAGTTTGTACAAAAAAGCAGGCTCC ATGGAAAGTAACCAATCGAACAATG
laf1 attB R	Primers for creating *laf-1* RNAi clone	GGGGACCACTTTGTACAAGAAAGCTGGGTC CTG AAG GGA AAG CTC ACG AG
CF418	MosSci insertion	TCTGGCTCTGCTTCTTCGTT
CF419	MosSci insertion	CAATTCATCCCGGTTTCTGT
oJL102	Mos1 element	CAACCTTGACTGTCGAACCACCATAG
oJL103	Mos1 element	TCTGCGAGTTGTTTTTGCGTTTGAG
mosSCI R outside	Right primer for mosSCI insertion validation outside	GGAGGCGAACCTAACTG
lin-41(n2914) f:	Genotyping lin-41(n2914) worm strain	CCTTTTCAGACTTGGAAAAAGTG
lin-41(n2914) r:	Genotyping lin-41(n2914) worm strain	CTGGTAGCATGATTGGCAC
lin-41 (n2914) mut rev:	Genotyping lin-41(n2914) worm strain	ATTGGCACGTTACAAACGAAC
qPCR mab-10 F1	QPCR primers	TCTCCGATTTTTGAGTCAGCTGT
qPCR mab-10 R1	QPCR primers	GAGAACTTGAACGCCAACGG
qPCR mab-3 F1	QPCR primers	ACAGAAATCCCGAGATGGTAAAGA
qPCR mab-3 R1	QPCR primers	GGACTTGCTGATGTTCCAATTATCT
qPCR dmd-3 F1	QPCR primers	CCGTCGCCGATAGATACAGT
qPCR dmd-3 R1	QPCR primers	GTTGGGCACACTTCAGACAC
qPCR act-1 F1	QPCR primers	GTTGCCCAGAGGCTATGTTC
qPCR act-1 R1	QPCR primers	CAAGAGCGGTGATTTCCTTC
qPCR lin-29 F1	QPCR primers	CCGACGAGTACGAAGAATGG
qPCR lin-29 R1	QPCR primers	GTGATTGTGGGTTGAACACG
qPCR lin-41 F2	QPCR primers	ACATCCTGGAAAGCATCGAG
qPCR lin-41 R2	QPCR primers	AAGCGTTGACGTGTGTATCG
qPCR daf-12 F3	QPCR primers	TTATATCCCGGCCACTCTCA
qPCR daf-12 R3	QPCR primers	TGGAACACCAGGTAACGACA
qPCR unc-54 F1	QPCR primers	CTGCTATGCTCATCTACACCT
qPCR unc-54 R1	QPCR primers	TGTGGTGGCATTTCTGTCTT

Table. 3	
Strain name	**Genotype**
***let-7* project strains**	
HW769	xeSi10*[Pwrt-2::gfp(PEST)-h2b::lin-41 3'UTR, unc-119 (+)] II*
HW896	xeSi10*[Pwrt-2::gfp(PEST)-h2b::lin-41 3'UTR, unc-119 (+)] II, let-7(n2853) X*
HW786	xeSi22*[Pwrt-2::GFP(PEST)-H2B::unc-54 3'UTR, unc-119 (+)] II*
HW899	xeSi22*[Pwrt-2::GFP(PEST)-H2B::unc-54 3'UTR, unc-119 (+)] II, let-7(n2853) X*
WM242	neSi12 *[cdk-1::gfp(+), cb-unc-119(+)] II; unc-119(ed3) III*
GR1434	wIs54*[scm::gfp]; let-7(n2853) V*
HW651	*let-7(n2853) V; maIs105 [col-19::gfp]*
HW1550	EG6699; xeSi185[*Pwrt-2::gfp(pest)/h2b::cdc-25.2 3'UTR*] II
HW1551	EG6699; xeSi186[*Pwrt-2::gfp(pest)/h2b::cdk-1 3'UTR*] II
HW1554	EG6699; xeSi185[*Pwrt-2::gfp(pest)/h2b::cdc-25.2 3'UTR*] II; *let-7(n2853ts)*
HW1555	EG6699; xeSi186[*Pwrt-2::gfp(pest)/h2b::cdk-1 3'UTR*] II; *let-7(n2853ts)*
HW1563	CDK-1::GFP *cb-unc-119(+)* II; *unc-119(ed3)* III; *let-7(n2853ts)*
HW4	*lin-29(n546)*
HW14	*let-7(n2853ts)*
LIN-41 project strains	
HW1564	EG6699; xeSi189[*Pdpy-30::gfp(pest)/h2b::lin-29 3'UTR*] II
HW1565	EG6699; xeSi190[*Pdpy-30::gfp(pest)/h2b::lin-29 3'UTR* Bashing1 with PAS site] II
HW1566	EG6699; xeSi191[*Pdpy-30::gfp(pest)/h2b::lin-29 3'UTR* Bashing2 with PAS site] II
HW1567	EG6699; xeSi192[*Pdpy-30::gfp(pest)/h2b::lin-29 3'UTR* Bashing3 with PAS site] II
HW1568	EG6699; xeSi193[*Pdpy-30::gfp(pest)/h2b::mab-10 3'UTR*] II
HW1569	EG6699; xeSi194[*Plin-29::gfp(pest)/h2b::lin-29 3'UTR*] II
HW1570	EG6699; xeSi195[*Plin-29::gfp(pest)/h2b::xrn-2 3'UTR*] II
HW1571	EG6699; xeSi196[*Plin-29::gfp(pest)/h2b::unc-54 3'UTR*] II
HW1572	EG6699; xeSi189[*Pdpy-30::gfp(pest)/h2b::lin-29 3'UTR]* II; *lin-41(n2914)/unc-29(e1072) lin-11(n1281)* I
HW1573	EG6699; xeSi197[*Plin-41::flag::gfp::lin-41::lin-41 3'UTR*] II
HW1574	EG6699; xeSi197[*Plin-41::flag::gfp::lin-41::lin-41 3'UTR] II; lin-41(n2914)*] I
HW1575	EG6699; xeSi198[*Plin-41::flag::gfp::lin-41(del NHL C302)::unc-54 3'UTR]* II
HW1576	EG6699; rrrSi??[*Plin-41::flag::gfp::lin-41(del NHL C302)::lin-41 3'UTR]* II
HW1577	EG6699; rrrSi??[*Plin-41::flag::gfp::lin-41(Y941A)::lin-41 3'UTR]* II
HW1578	EG6699; rrrSi??[*Plin-41::flag::gfp::lin-41(Y941A)::lin-41 3'UTR*] II; rrr3
HW1606	*lin-41(tn1491) I/hT2[bli-4(e937) let-?(q782) qIs48] (I;III)* Sterile balanced mutant in B box (Premature stop codon)
HW1607	*lin-41(tn1496) I/hT2[bli-4(e937) let-?(q782) qIs48] (I;III)* Sterile balanced mutant in C-coil (Premature stop codon)
HW1608	*lin-41(tn1492)* I (viable miss-sense mutation in last NHL domain repeat [T1124I])
HW1609	*lin-41(tn1487*ts*)* I (Temperature sensitive mutant in last NHL domain repeat [D1125N])
HW1610	*lin-41(tn1487tn1515)* I (E1028K intragenic suppressor in NHL repeat between 4th NHL and 5th NHL repeat)
HW1611	*lin-41(tn1487tn1516)* I (C976Y intragenic suppressor in NHL repeat between 3rd and 4th NHL repeat)
HW1612	*lin-41(tn1487 tn1536)* I (E375K intragenic suppressor in B box)
HW1613	*lin-41(tn1487 tn1539)* I (E388K intragenic suppressor in B box)
HW1614	*lin-41(tn1541[gfp::tev::s::lin-41])* I (tagged lin-41 line)
HW1615	*lin-41(tn1505)*/hT2(qIs48)
HW763 ("MT7897 from CGC")	*lin-41(n2914)/unc-29(e1072) lin-11(n1281)* I
HW7	*lin-41(ma104)*
HW12	*let-7(mn112); lin-28(xxx); unc-3(xxx)*
B-Box Domain mutant	*lin-41(rrr3) (I), rrrsi308[plin-41-::FLAG-GFP-C479S, C490S, C493S, C500S, C503S_LIN-41::lin-41 3'UTR; unc-119(+)]II*
RING Domain mutant	*lin-41(rrr3) (I), rrrsi308[plin-41-::FLAG-GFP-C112S, C117S, C130S, C151S, C154S_LIN-41::lin-41 3'UTR; unc-119(+)]II*
	lin-41(tn1505) I/hT2[bli-4(e937) let-?(q782) qIs48] (I;III) Sterile balanced mutant before RING domain (Premature stop codon)
	EG6699; xeSi??[*Plin-29::mcherry(pest)/h2b::lin-29 3'UTR*] I
	lin-41(tn1491) I/hT2[bli-4(e937) let-?(q782) qIs48] (I;III) Sterile balanced mutant in B box (Premature stop codon); *xeSi194[Plin-29::gfp(pest)/h2b::lin-29 3'UTR] II*
	lin-41(tn1496) I/hT2[bli-4(e937) let-?(q782) qIs48] (I;III) Sterile balanced mutant in C-coil (Premature stop codon); *xeSi194[Plin-29::gfp(pest)/h2b::lin-29 3'UTR] II*
	lin-41(tn1492) I (viable miss-sense mutation in last NHL domain repeat [T1124I])
	*lin-41(tn1487*ts*)* I (Temperature sensitive mutant in last NHL domain repeat [D1125N]); *xeSi194[Plin-29::gfp(pest)/h2b::lin-29 3'UTR] II*
	lin-41(tn1487tn1515) I (E1028K intragenic suppressor in NHL repeat between 4th NHL and 5th NHL repeat); *xeSi194[Plin-29::gfp(pest)/h2b::lin-29 3'UTR] II*
	lin-41(tn1487tn1516) I (C976Y intragenic suppressor in NHL repeat between 3rd and 4th NHL repeat); *xeSi194[Plin-29::gfp(pest)/h2b::lin-29 3'UTR] II*
	lin-41(tn1487 tn1536) I (E375K intragenic suppressor in B box); *xeSi194[Plin-29::gfp(pest)/h2b::lin-29 3'UTR] II*
	lin-41(tn1487 tn1539) I (E388K intragenic suppressor in B box); *xeSi194[Plin-29::gfp(pest)/h2b::lin-29 3'UTR] II*
	lin-41(rrr3) (I), rrrsi308[plin-41-::FLAG-GFP-C112S, C117S, C130S, C151S, C154S_LIN-41::lin-41 3'UTR; unc-119(+)]II; *xeSi??[Plin-29::mcherry(pest)/h2b::lin-29 3'UTR] I*
	EG6699; rrrSi??[*Plin-41::flag::gfp::lin-41(Y941A)::lin-41 3'UTR]* II; *xeSi??[Plin-29::mcherry(pest)/h2b::lin-29 3'UTR] I*

Table. 4	
Plasmid	
Name	**Purpose**
pENTR_L4-R1_Pdpy-30	*dpy-30* promoter GW entry clone covering the V:12189538- 12191540 genomic region
pBMF2.7	Gfp(PEST)-H2b GW enty clone,
pCM1.151	mCherry-H2b GW enty clone,
pCM5.37	GW entry clone for *unc-54* 3'UTR
pENTR_R2-L3_lin-41 3'UTR	GW entry clone *lin-41* 3'UTR
pENTR_L4-R1_Plin-41	GW entry clone *lin-41* promoter
pENTR_L1-L2_lin-41flaggfp	GW clone for C terminally flag gfp tagged *lin-41* transgene
pENTR_L1-L2_flaggfplin-41	GW clone for N terminally flag gfp tagged *lin-41* transgene
pENTR_L4-R1_Plin-29	GW entry clone for *lin-29* promoter
pENTR_R2-L3_xrn-2 3'UTR	GW entry clone for *xrn-2* 3'UTR
pENTR_R2-L3_lin-29 3'UTR	GW entry clone for *lin-29* 3'UTR
pENTR_R2-L3_cdc-25.2 3'UTR	GW entry clone for *cdc-25.2* 3'UTR
pENTR_R2-L3_cdk-1 3'UTR	GW entry clone for *cdk-1* 3'UTR
pCFJ104	Pmyo-3::mCherry red marker body wall muscles
pGH8	Prab-3::mCherry red marker for nervous system
pCFJ90	Pmyo-2::mCherry red marker for pharynx
pCFJ601	catalyzes Mos1 mobilization
pCFJ150	Gateway vector from Chr II insertion
pCFJ210	Gateway vector from Chr I insertion
L4440	RNAi vector cloning

10. Supplementary Figure. 1

Supp. Figure. 1.1 LIN-41 Co-IP using lysate from mixed stage worms (IP number-3)

Accession	Peptides	Score	Fold	Tags	Description	Average Normalised	
						S01 Sart	S02 Lin41
LIN41_CAEEL	21	1303.39	63.93		Isoform a of Protein lin-41 GN=lin-41	1.29E+04	8.28E+05
H2L048_CAEE	7	330.45	29.31		Protein F46H5.7, isoform a GN=CELE_F46H5.7	2500.77	7.33E+04
YP83_CAEEL	2	138.23	24.04		ARID domain-containing protein C08B11.3 GN=C08B11.3	996.71	2.40E+04
Q19991_CAEEL	5	219.72	21.21		Protein F33H1.4 GN=CELE_F33H1.4	3189.95	6.77E+04
Q21090_CAEEL	2	157.41	16.71		Protein K01D12.1 GN=K01D12.1	2479.36	4.14E+04
ANC1_CAEEL	5	258.15	16.1		Nuclear anchorage protein 1 GN=anc-1	1591.67	2.56E+04
CGH1_CAEEL	12	603.4	10.49		ATP-dependent RNA helicase cgh-1 GN=cgh-1	2.95E+04	3.10E+05
RENT1_CAEEL	19	1001.13	8.19		Regulator of nonsense transcripts 1 GN=smg-2	2.84E+04	2.32E+05
Q9N4N4_CAEEL	2	110.27	7.84		Protein SWSN-6 GN=swsn-6	4178.84	3.28E+04
Q9XW17_CAEEL	11	887.42	7.76		Protein CAR-1 GN=car-1	9.62E+04	7.46E+05
Q9U302_CAEEL	7	432.23	7.58		Protein PAB-1, isoform a GN=pab-1	1.19E+04	9.00E+04
CH60_CAEEL	33	3238.13	5.53		Chaperonin homolog Hsp-60, mitochondrial GN=hsp-60	1.32E+06	7.32E+06

	2 or more
	>50 confidence score
	highest lin41
	>5 max fold change

Supp. Figure. 1.2 LIN-41 Co-IP using lysate from mixed stage worms (IP number-5) + RNAse treatment

Accession	Peptides	Score	Fold	Tags	Description	Average Normalised Abundances			
						S01 sart3	S02 sart3 RNase	S03 lin41	S04 lin41 RNase
LIN41_CAEEL	12	443.21	106.68		(Q9U489) Protein lin-41 GN=lin-41	9510.2	5565.83	3.52E+05	5.94E+05
ALF2_CAEEL	3	84.38	76.97		(P46563) Fructose-bisphosphate aldolase 2 GN=aldo-2	1098.22	2.96E+04	8.45E+04	1.19E+04
H2L048_CAEEL	6	132.01	42.03		(H2L048) Protein F46H5.7, isoform a GN=CELE_F46H5.7	1192.19	2864.2	3.17E+04	5.01E+04
KARG1_CAEEL	4	78.93	16.16		(Q10454) Probable arginine kinase F46H5.3 GN=F46H5.3	3751.53	1.18E+04	6.06E+04	1.11E+04
P91398_CAEEL	4	109.19	15.39		(P91398) Protein CEY-3 GN=cey-3	6.58E+04	6797.75	1.05E+05	9668.41
P91306_CAEEL	8	273.18	12.83		(P91306) Protein CEY-2 GN=cey-2	1.68E+05	2.06E+04	2.65E+05	2.17E+04
RL10A_CAEEL	7	354.27	11.78		(Q9N4I4) 60S ribosomal protein L10a GN=rpl-10a	4.27E+05	6.27E+04	7.39E+05	6.69E+04
ENO_CAEEL	3	71.47	11.46		(Q27527) Enolase GN=enol-1	1.01E+04	3905.13	4.48E+04	2.13E+04
DKC1_CAEEL	3	68.79	10.15		(O17919) Putative H/ACA ribonucleoprotein complex subunit 4 GN=K01G5.5	1218.62	2238.1	1.24E+04	4066.68
RL44_CAEEL	2	42.23	8.65		(P48166) 60S ribosomal protein L44 GN=rpl-41	6.14E+04	1.22E+04	1.05E+05	2.39E+04
G3P2_CAEEL	3	76.69	7.87		(P17329) Glyceraldehyde-3-phosphate dehydrogenase 2 GN=gpd-2	6818.35	1.79E+04	5.37E+04	2.20E+04
Q7K797_CAEEL	3	114.17	7.48		(Q7K797) Protein PAB-1, isoform c GN=pab-1	1.78E+04	6202.24	4.64E+04	3.55E+04
RL3_CAEEL	12	427.33	7.16		(P50880) 60S ribosomal protein L3 GN=rpl-3	1.81E+05	4.95E+04	3.54E+05	9.98E+04
Q9N5B3_CAEEL	5	214.89	6.65		(Q9N5B3) Protein W08E12.7 GN=CELE_W08E12.7	5.32E+04	1.76E+04	1.17E+05	3.15E+04
RL13A_CAEEL	3	98.33	6.59		(Q27389) 60S ribosomal protein L13a GN=rpl-16	3.63E+04	1.21E+04	7.94E+04	3.61E+04
RL9_CAEEL	7	322.55	6.2		(Q95Y90) 60S ribosomal protein L9 GN=rpl-9	5.05E+05	1.78E+05	1.10E+06	2.53E+05
RL24_CAEEL	3	105.37	6.03		(O01868) 60S ribosomal protein L24 GN=rpl-24.1	1.27E+05	5.34E+04	3.22E+05	1.05E+05
RS16_CAEEL	8	283.39	6.02		(Q22054) 40S ribosomal protein S16 GN=rps-16	4.00E+05	1.49E+05	8.99E+05	2.90E+05
H2KYR1_CAEEL	5	282.01	5.97		(H2KYR1) Protein VIG-1, isoform a GN=vig-1	2.97E+05	9.11E+04	5.44E+05	1.10E+05
RL18_CAEEL	7	405.57	5.82		(O45946) 60S ribosomal protein L18 GN=rpl-18	5.54E+05	1.65E+05	9.61E+05	2.92E+05
RLA0_CAEEL	13	556.19	5.73		(Q93572) 60S acidic ribosomal protein P0 GN=rpa-0	1.05E+06	3.56E+05	2.04E+06	5.44E+05
G5EDV3_CAEEL	8	228.78	5.72		(G5EDV3) Protein CEY-4 GN=cey-4	5.43E+04	2.67E+04	1.53E+05	4.15E+04
RL22_CAEEL	3	172.82	5.71		(P52819) 60S ribosomal protein L22 GN=rpl-22	3.55E+05	9.75E+04	5.57E+05	1.56E+05
RL21_CAEEL	5	187.23	5.61		(P34334) 60S ribosomal protein L21 GN=rpl-21	1.95E+05	8.75E+04	4.91E+05	1.07E+05
RS23_CAEEL	3	140.76	5.6		(Q19877) 40S ribosomal protein S23 GN=rps-23	1.82E+05	8.33E+04	4.66E+05	1.39E+05
RS3_CAEEL	13	538.2	5.56		(P48152) 40S ribosomal protein S3 GN=rps-3	4.65E+05	1.61E+05	8.97E+05	1.94E+05
MYO1_CAEEL	2	41.98	5.5		(P02567) Myosin-1 GN=let-75	1782.73	3517.49	9807.4	6187.02
ANC1_CAEEL	6	161.47	5.49		(Q9N4M4) Nuclear anchorage protein 1 GN=anc-1	1.20E+04	1.07E+04	5.86E+04	4.28E+04
Q9U1X9_CAEEL	2	107.45	5.47		(Q9U1X9) Protein RLA-2 GN=rla-2	2.32E+05	6.33E+04	3.46E+05	1.28E+05
A3QMC5_CAEEL	3	103.91	5.47		(A3QMC5) Protein RPL-34 GN=rpl-34	1.73E+05	4.81E+04	2.63E+05	7.17E+04
RL13_CAEEL	7	259.23	5.44		(P91128) 60S ribosomal protein L13 GN=rpl-13	4.52E+05	1.41E+05	7.66E+05	2.37E+05
I2HAJ2_CAEEL	2	91.38	5.43		(I2HAJ2) Protein RPL-30, isoform c GN=rpl-30	2.13E+05	5.24E+04	2.85E+05	8.05E+04
RL10_CAEEL	9	270.54	5.41		(Q09533) 60S ribosomal protein L10 GN=rpl-10	2.25E+05	6.09E+04	3.30E+05	1.06E+05
BTF3_CAEEL	4	174.83	5.34		(Q18885) Transcription factor BTF3 homolog GN=icd-1	2.00E+05	1.12E+05	5.96E+05	1.15E+05
RS8_CAEEL	6	285.54	5.3		(P48156) 40S ribosomal protein S8 GN=rps-8	3.20E+05	1.20E+05	6.34E+05	2.35E+05
RL4_CAEEL	13	518.53	5.3		(O02056) 60S ribosomal protein L4 GN=rpl-4	1.49E+06	4.69E+05	2.49E+06	6.34E+05
Q9XW17_CAEEL	8	253.7	5.03		(Q9XW17) Protein CAR-1 GN=car-1	6.96E+04	5.41E+04	2.12E+05	2.72E+05

Tags	
	confidance>40
	fold change>5
	1peptide only
	highest in Lin41 S3 or S4
	lowest in S4 lin RNase
	lowest in S3 lin

Supp. Figure. 1.3 LIN-41 Co-IP using lysate from mixed stage worms (IP number-6)

Accession	Peptides	Score	Fold	Tags	Description	Average Normalised	
						Lin	sart
LIN41_CAEEL	24	1497.15	20.13		(Q9U489) Protein lin-41 GN=lin-41	1.02E+06	5.07E+04
Q9XW17_CAEEL	13	888.37	7.77		(Q9XW17) Protein CAR-1 GN=car-1	3.14E+05	4.04E+04
H2L048_CAEEL	9	385.71	13.18		(H2L048) Protein F46H5.7, isoform a GN=CELE_F46H5.7	3.95E+04	2999.71
Q18943_CAEEL	4	269.4	17.54		(Q18943) Protein D1054.10 GN=CELE_D1054.10	7.31E+04	4166.04
Q19007_CAEEL	6	244.54	14.16		(Q19007) Protein NAP-1 GN=nap-1	2.79E+04	1968.41

O44144_CAEEL	3	183.2	6.48		(O44144) Protein PERM-4 GN=perm-4	3.00E+04	4633.1
O44145_CAEEL	2	166.71	6.64		(O44145) Protein PERM-2 GN=perm-2	2.99E+04	4503.87
PDIA6_CAEEL	2	145.52	5.46		(Q11067) Probable protein disulfide-isomerase A6 GN=tag-320	2086.95	382.26
O16303_CAEEL	3	141.75	11.14		(O16303) Protein DNJ-19 GN=dnj-19	1.48E+04	1325.51
ANC1_CAEEL	2	106.78	38.43		(Q9N4M4) Nuclear anchorage protein 1 GN=anc-1	6224.2	161.96
Q9XWT3_CAEEL	2	100.06	45.8		(Q9XWT3) Protein Y62H9A.6 GN=CELE_Y62H9A.6	5595.8	122.18

Tags	
	<50 confidence
	highest in lin41
	highest in sart3
	2 or more peptides
	>5xmax fold change
	<100 confidence

Supp. Figure. 1.4 LIN-41 Co-IP using lysate from larval stage 3 worms (IP number-7)

Accession	Peptides	Score	Fold	Tags	Description	Average Normalised	
						Control	lin41
LIN41_CAEEL	46 (45)	2819.04	120.13		(Q9U489) Protein lin-41 GN=lin-41	2.96E+04	3.55E+06
DMON2_CAEEL	2	68.56	62.71		(Q9XWC2) DOMON domain-containing protein Y73F4A.1 GN=Y73F4A.1	2.28E+04	2.19E+05
Q9NA78_CAEEL	5	196.84	54.17		(Q9NA78) Protein Y57A10A.23 GN=CELE_Y57A10A.23	1374.17	7.44E+04
D0PV95_CAEEL	2	109.91	14.1		(D0PV95) Protein LAF-1, isoform b GN=laf-1	7983.24	4.87E+04
MYO4_CAEEL	24 (19)	1118.77	9.59		(P02566) Myosin-4 GN=unc-54	8446.17	4.35E+04
Q9TZS5_CAEEL	2	57.52	7.15		(Q9TZS5) Protein CCT-7, isoform a GN=cct-7	951.17	1.34E+04
Q95YC7_CAEEL	4	166.33	6.11		(Q95YC7) Protein C45B2.2 GN=C45B2.2	1586.14	8512.95
Q9N5S7_CAEEL	2	107.76	5.37		(Q9N5S7) Protein F49H12.5 GN=CELE_F49H12.5	175.96	1.10E+04
Q95YD8_CAEEL	3 (2)	132.24	5.15		(Q95YD8) Protein IDHG-2 GN=idhg-2	2818.16	2.01E+04

	2 or more peptides
	>50 confidence score
	highest lin41
	>5 max fold change

10. Supplementary Table. 1

Predicted genes	Wormbase ID	Predicted genes	Wormbase ID	Predicted genes	Wormbase ID
Lin-41 CoIP hits		**Suppressors of *let-7(n2853)* lethal bursting phenotype**		**Suppressors of *let-7(n2853)* lethal bursting phenotype**	
laf-1	WBGene00002244	*R153.1*	WBGene00020114	*T09B4.9*	WBGene00020383
sqd-1	WBGene00022235	*Y51H7B_5.b*	WBGene00022042	*W01B11.3*	WBGene00020915
cgh-1	WBGene00000479	*ZK430.1*	WBGene00022739	*Y48G1A_54.d*	WBGene00021660
pab-1	WBGene00003902	*T12C9.2*	WBGene00022739	*Y54E10A.10*	WBGene00021830
smg-2	WBGene00004880	*ZK430.7*	WBGene00022742	*Y54E10BR.5*	WBGene00021844
swsn-7	WBGene00007433	*ZK1127.5*	WBGene00022852	*Y54E10B_159.c*	WBGene00021845
F46H5.7	WBGene00018522	*F54H12.1*	WBGene00000041	*Y65B4B_10.d*	WBGene00022042
nmy-1	WBGene00003776	*F57B9.5*	WBGene00000276	*Y110A7A.m*	WBGene00022458
car-1	WBGene00012484	*T05G5.3*	WBGene00000405	*ZC581.1*	WBGene00022631
rpn-1	WBGene00004458	*R08D7.3*	WBGene00001227	*ZC581.1*	WBGene00022631
copb-1	WBGene00021292	*W07B3.2*	WBGene00001561	*M106.5*	WBGene00000293
copb-2	WBGene00009542	*T20B12.8*	WBGene00001974	*R06F6.1*	WBGene00000411
hcp-1	WBGene00001829	*Y22D7AL.5*	WBGene00002025	*F33A8.3*	WBGene00000472
T10B5.5	WBGene00020391	*C02F5.1*	WBGene00002231	*F11G11.10*	WBGene00000606
C45B2.2	WBGene00016659	*C16A3.3*	WBGene00002850	*R53.3*	WBGene00001207
F49H12.5	WBGene00018656	*C29E4.8*	WBGene00002879	*M110.4*	WBGene00002066
C30F12.7	WBGene00016266	*C07H6.7*	WBGene00003024	*Y17G7A.2*	WBGene00003015
Y57A10A.23	WBGene00013263	*R13A5.12*	WBGene00003063	*Y17G7B.5*	WBGene00003154
Y73F4A.1	WBGene00013514	*ZK632.1*	WBGene00003158	*R06F6.10*	WBGene00003367
anc-1	WBGene00000140	*F59A2.1*	WBGene00003795	*K12D12.2*	WBGene00003789
		K06H7.1	WBGene00004042	*K01C8.9*	WBGene00003821
Additional factors tested		*C14B9.4*	WBGene00004042	*F10C1.5*	WBGene00004015
let-711	WBGene00002845	*F58A4.4*	WBGene00004180	*W07E6.4*	WBGene00004188
unc-54	WBGene00006789	*F58A4.4*	WBGene00004180	*ZK1290.3*	WBGene00004398
ain-1	WBGene00015547	*K04G7.10*	WBGene00004390	*C09H10.2*	WBGene00004454
ain-2	WBGene00015007		WBGene00004391	*C08B11.5*	WBGene00004723
alg-1	WBGene00000105	*C54C6.1*	WBGene00004451	*B0491.2*	WBGene00005016
alg-2	WBGene00000106	*Y47D3A.26*	WBGene00004873	*C01B12.1*	WBGene00005017
ccf-1	WBGene00000369	*F35G12.8*	WBGene00004874	*F10G7.1*	WBGene00006497
ccr-4	WBGene00000376	*ZK652.1*	WBGene00004918	*C56E6.1*	WBGene00006522
ntl-2	WBGene00003825	*T27E9.1*	WBGene00006439	*D2085.3*	WBGene00008428
ntl-3	WBGene00003826	*T27E9.1*	WBGene00006439	*F44G4.1*	WBGene00009711
ntl-4	WBGene00003827	*Y92C3B.2*	WBGene00006697	*R03D7.1*	WBGene00010988
ntl-9	WBGene00016139	*R10E11.2*	WBGene00006911	*W03H9.4*	WBGene00012230
		Y111B2C.m	WBGene00007030	*Y48B6A.1*	WBGene00012978
Suppressors of *let-7(n2853)* lethal bursting phenotype		*Y111B2A.11*	WBGene00007030	*C01F1.3*	WBGene00015298
Y54E10A_156.a	WBGene00000413	*M01F1.7*	WBGene00010813	*F40H3.5*	WBGene00015941
C03D6.1	WBGene00000466	*T04A8.6*	WBGene00011408	*C18A3.3*	WBGene00015941
B0511.10	WBGene00001228		WBGene00012059	*C44B7.3*	WBGene00016625
B0511.10	WBGene00001228		WBGene00012936	*F18A1.5*	WBGene00017546
C41D11.2	WBGene00001231	*Y49E10.19*	WBGene00013038	*F47F6.1*	WBGene00018572
C47B2.5	WBGene00001234	*C16A3.4*	WBGene00015809	*F47F6.2*	WBGene00018572
T08B2.9	WBGene00001497	*C16A3.6*	WBGene00015811	*F54A3_31.e*	WBGene00018782
ZK484.2	WBGene00001819	*C23G10.8*	WBGene00016015	*C32D5.11*	WBGene00018869
T03F1.9	WBGene00001832	*C23G10.9*	WBGene00016015	*C17G1.6*	WBGene00003553
Y110A7A.1	WBGene00001833	*C29E4.2*	WBGene00016202	*F11C1.6*	WBGene00003623
F28B3.7	WBGene00001860	*F09F7.3*	WBGene00017300	*C49F5.1*	WBGene00008205
Y48G1A_54.b	WBGene00002079	*F26F4.11*	WBGene00017830	*F29G9.3*	WBGene00000159
Y48G1A_54.c	WBGene00002079	*F37C12.13*	WBGene00018154	*K11C4.5*	WBGene00000159
C01G8.7	WBGene00002717	*H06I04.3*	WBGene00019168	*F29G9.3*	WBGene00000159
C01G8.8	WBGene00002717	*H06I04.3*	WBGene00019168	*K07C5.1*	WBGene00000200
F57B10.1	WBGene00002783	*K12H4.3*	WBGene00019678	*F16B4.8*	WBGene00000387
C12C8.3	WBGene00003026	*K12H4.4*	WBGene00019679	*T06E6.2*	WBGene00000868
T19A6.2	WBGene00003596	*F57H12.1*	WBGene00000183	*F29G9.4*	WBGene00001345
F56A3.3	WBGene00003792	*Y41E3.2*	WBGene00001066	*C53A5.3*	WBGene00001834
T19B4.2	WBGene00003793	*F35H10.1*	WBGene00001904	*ZK742.1*	WBGene00002078
Y71F9A_279.b	WBGene00003836	*T11G6.1*	WBGene00002001	*Y73C8B.b*	WBGene00002246
Y106G6H.2	WBGene00003902	*F32E10.4*	WBGene00002074	*C29A12.3*	WBGene00002985
W02D9.1	WBGene00004181	*M7.1*	WBGene00002344	*F32D1.10*	WBGene00003159
C03D6.8	WBGene00004437	*F36H1.4*	WBGene00002992	*F38A6.1*	WBGene00004013
K12C11.2	WBGene00004888	*C08F8.8*	WBGene00003657	*F18E2.3*	WBGene00004738
F56A3.4	WBGene00004955	*Y41D4A_3073.a*	WBGene00003794	*F23H12.4*	WBGene00005018
Y63D3A.5	WBGene00006565	*Y41D4A_3073.b*	WBGene00003794	*D1054.15*	WBGene00006481
C36B1.3	WBGene00007971	*Y41D4A_3457.a*	WBGene00003794	*Y80D3A.a*	WBGene00006945
Y53C10A.3	WBGene00008670	*Y41D4A_3457.d*	WBGene00003794	*B0250.7*	WBGene00007124
F14B4.3	WBGene00008781	*T23F6.4*	WBGene00004315	*C14C10.3*	WBGene00007586
F14B4.3	WBGene00008781	*Y62E10A.d*	WBGene00004410	*C15H11.9*	WBGene00007617
M04C9.6	WBGene00008878	*C42D4.8*	WBGene00004411	*C27H6.2*	WBGene00007784
F20G4.1	WBGene00008990	*K11H12.12*	WBGene00004427	*F11A3.2*	WBGene00008670
F58H10.1	WBGene00010291	*K08E4.1*	WBGene00005015	*C50F4.13*	WBGene00008670
K04G2.1	WBGene00010560	*M03D4.1*	WBGene00006974	*F53B7.3*	WBGene00009966
K07A1.2	WBGene00010609	*C33D9.3*	WBGene00007898	*F53F4.11*	WBGene00009993
R06C7.5	WBGene00011064	*C47E12.4*	WBGene00008149	*F53F4.11*	WBGene00009993
T23D8.3	WBGene00011944	*C47E12.7*	WBGene00008151	*F55C5.4*	WBGene00010093
W04A4.6	WBGene00012234	*F28D1.1*	WBGene00009211	*F55C5.8*	WBGene00010097
W06H12.1	WBGene00012317	*F40F11.2*	WBGene00009587	*K07C5.4*	WBGene00010627
Y105E8C.d	WBGene00013676	*M18.5*	WBGene00010890	*T06E6.1*	WBGene00011538
B0511.6	WBGene00015232	*T11G6.8*	WBGene00011722	*C37H5.5*	WBGene00016508
C43E11.9	WBGene00016607	*Y45F10D.8*	WBGene00012887	*F09G2.4*	WBGene00017313
C53H9.2	WBGene00016907	*C42C1.3*	WBGene00016581	*F25G6.2*	WBGene00017797
F55A12.8	WBGene00018866	*H06H21.3*	WBGene00019162	*F32D1.2*	WBGene00017982
F55F8.3	WBGene00018891	*T22D1.10*	WBGene00020687	*T08B1.1*	WBGene00020340
H27M09.2	WBGene00019246	*Y55F3A_750.d*	WBGene00021934	*T19A5.3*	WBGene00020554
K06A5.4	WBGene00019432	*Y55F3A_750.e*	WBGene00021934	*F08C6.1*	WBGene00000083
C42D8.8	WBGene00000149	*C02C6.1*	WBGene00001130	*F52D10.3*	WBGene00001502
F18H3.5	WBGene00000406	*W01C8.2*	WBGene00001182	*F13D11.2*	WBGene00001824
F11A1.3	WBGene00000908	*C33D3.1*	WBGene00001250	*F46F2.2*	WBGene00002203

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A genetic interactome of the *let-7* microRNA in *C. elegans*

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ABSTRACT

The heterochronic pathway controls temporal patterning during *Caenorhabditis elegans* larval development. The highly conserved *let-7* microRNA (miRNA) plays a key role in this pathway, directing the larval-to-adult (L/A) transition. Hence, knowledge of the genetic interactome of *let-7* has the potential to provide insight into both control of temporal cell fates and mechanisms of regulation and function of miRNAs. Here, we report the results of a genome-wide, RNAi-based screen for suppressors of *let-7* mutant vulval bursting. The 201 genetic interaction partners of *let-7* thus identified include genes that promote target silencing activity of *let-7*, seam cell differentiation, or both. We illustrate the suitability of our approach by uncovering the mitotic cyclin-dependent kinase CDK-1 as a downstream effector of *let-7* that affects both seam cell proliferation and differentiation, and by identifying a core set of candidate modulators of *let-7* activity, which includes all subunits of the condensin II complex. We propose that the genes identified in our screen thus constitute a valuable resource for studies of the heterochronic pathway and miRNAs.

Introduction

Proper organismal development requires faithful temporal and spatial control of gene expression. In the nematode *Caenorhabditis elegans*, the heterochronic pathway controls temporal patterning during larval development by ensuring successive occurrence of specific developmental programs in distinct tissues at the correct time (Ambros and Horvitz, 1984). Heterochronic mutations may thus cause retarded phenotypes, where developmental events characteristic of one larval stage are reiterated during subsequent stages, or precocious phenotypes, where stage-specific programs are skipped in favor of subsequent programs.

A classical example of a developmental process controlled by the heterochronic pathway is the establishment of the adult *C. elegans* hypodermis (skin), which mainly consists of the large multinuclear hyp7 syncytium as well as two sets of lateral hypodermal blast cells called seam cells (Sulston et al., 1983; Podbilewicz and White, 1994). The seam cells are characterized by a stem cell-like, asymmetric division during larval stages that, in most lineages, generates posterior daughters that maintain the proliferative potential and anterior daughters that differentiate and fuse to the hypodermal syncytium (Sulston and Horvitz, 1977). This mechanism allows elongation of the hypodermis proportional to the growth in body size during larval development. Upon transition from larval to adult stage, seam cells cease proliferation and terminally differentiate, i.e., they fuse into a syncytium and express adult-specific collagens to generate an adult cuticular structure known as alae (Singh and Sulston, 1978). These events depend on the *let-7* microRNA, which accumulates strongly during

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0012-1606/

the last larval (L4) stage (Reinhart et al., 2000). *let-7* exerts its function by binding to partially complementary sequences in the 3′ untranslated regions (3′ UTRs) of target mRNAs, which silences these through inhibition of their translation or through degradation (Slack et al., 2000, Lin et al., 2003; Abrahante et al., 2003; Großhans et al., 2005; Ding and Großhans, 2009; Bagga et al., 2005). Loss of *let-7* activity leads to failed silencing of its targets and, consequently, continued seam cell proliferation, failed fusion, and sustained expression of larval- instead of adult-specific cuticular collagens (Reinhart et al., 2000). *let-7* mutant animals also display a vulval rupturing phenotype that causes their death (Reinhart et al., 2000), but it is currently unclear if and to what extent this is linked to the retarded heterochronic seam cell phenotypes (Roush and Slack, 2008; Ecsedi et al., 2015).

The sequence of *let-7* is invariant across animal phylogeny (Pasquinelli et al., 2000), and a number of targets are conserved (Slack et al., 2000; Großhans et al., 2005). Indeed, function in inhibition of proliferation and induction of differentiation is a common feature of *let-7* from invertebrates to mammals (Büssing et al., 2008). Thus, *let-7* suppresses self-renewal of embryonic stem cells, promotes neural stem cell differentiation, and acts as a tumor suppressor gene (Takamizawa et al., 2004; Melton et al., 2010; Worringer et al., 2014; Rybak et al., 2008). These functions may involve regulation of a number of direct *let-7* targets, including oncogenes such as *MYC*, *RAS*, and *HMGA2*, but also cell cycle genes such as CDK6 and CDC25A (Johnson et al., 2007; Lee and Dutta, 2007; Sampson et al., 2007; Johnson et al., 2005).

For *C. elegans let-7*, previously identified direct targets include the TRIM-NHL protein LIN-41 (Slack et al., 2000), and the transcription factors DAF-12 (Großhans et al., 2005) and HBL-1 (Lin et al., 2003; Abrahante et al., 2003). In addition, genetic data revealed that hypodermal LIN-41 represses, directly or indirectly, accumulation of the zinc finger transcription factor LIN-29 (Slack et al., 2000), which in turn is needed for expression of the adult-specific collagen *col-19* and the cell cycle inhibitor *cki-1* (Rougvie and Ambros, 1995; Liu et al., 1995; Hong et al., 1998). Hence, *let-7* may promote at least some aspects of the L/A transition by relieving LIN-29 from LIN-41-mediated repression. Whether it additionally exerts direct repression of cell cycle genes is currently unknown.

Here, we conducted a genome-wide study for genetic interactors of *let-7*. The purpose of this study was two-fold. First, we sought to identify downstream effectors to obtain a better understanding of *let-7* function in the heterochronic pathway. Second, we aimed to establish a genome-wide collection of modulators of *let-7* activity to identify candidate components of the miRNA pathway (Ding et al., 2008; Hunter et al., 2013; Großhans et al., 2005; Büssing et al., 2010; Parry et al., 2007). We illustrate the suitability of our approach for these purposes by identifying 201 suppressors of *let-7* mutant vulval bursting, establishing the mitotic cyclin-dependent kinase CDK-1 as a downstream effector of *let-7*, and uncovering a core set of candidate modulators of *let-7* activity that include all subunits of the condensin II complex.

Materials and methods

A genome-wide RNAi screen for suppressors of let-7(n2853) bursting

RNAi by feeding (Timmons et al., 2001) was performed using primarily the RNAi library from the Ahringer group (Kamath et al., 2003) supplemented with unique clones from the Vidal library (Rual et al., 2004). The two libraries together are predicted to target 18′578 loci representing ~94% of *C. elegans* protein coding genes (Kim et al., 2005). L1 stage *let-7(n2853)* worms synchronized by hatching overnight in M9 buffer were grown in 96-well plates at a concentration of 25 worms per well in S-medium liquid culture with RNAi bacteria; double-stranded RNA production was induced by IPTG (4 mM final concentration in the bacterial growth medium). Wells were scored for surviving adult worms after 70 h of incubation at 25 °C using a dissecting microscope. *let-7(n2853)* animals grown on mock RNAi showed a >90% penetrant bursting phenotype under these conditions. Bacteria from positive wells were streaked directly from the wells, and a single colony was selected for retesting on RNAi plates at 20 °C and 25 °C as described previously (Ding et al., 2008). For clones scoring positive again, the RNAi plasmid was isolated, sequenced and retransformed into *HT115* bacteria. This new library of positive clones was retested on RNAi plates at 20 °C and 25 °C. Bursting suppression was scored as indicated in the legend of Table S1.

col-19::gfp assay

col-19::*gfp*; *let-7(n2853)* worms ($n > 100$) were tested at 20 °C and 25 °C on suppressor RNAi plates as in the bursting suppressor screen. Worms were scored at two time points (48 h and 56 h for 25 °C and 56 h and 72 h, respectively, for 20 °C) for presence of detectable GFP expression in the hypodermis using a Leica MZ16 FA fluorescence dissection microscope. At the magnification used, it was not possible to differentiate between expression in hyp7 or seam cell nuclei. As *let-7(n2853)* worms, at the permissive temperature of 15 °C, undergo a larval-to-adult transition after an L5 molt and eventually express *col-19::gfp*, we scored suppressors based both on the penetrance and timing of *col-19::gfp* expression as indicated in the legend of Table S3. Certain suppressors (results) were examined further on a Zeiss Z-1 microscope and imaged with Zeiss Axiovision software.

let-7 target and cdc-25.2 and cdk-1 3′UTR reporters

The hypodermal-specific *wrt-2* promoter (Aspöck et al., 1999) and indicated 3′UTRs were amplified using the primers listed in the supplementary methods and inserted into an appropriate Gateway donor vector. *Pwrt-2*, *gfp::h2b::PEST* (pBMF2.7) and individual 3′UTR entry vectors were recombined into the MosSCI-compatible pCFJ150 plasmid. All plasmids were verified by sequencing. Transgenes were integrated in single copy at a defined genomic location as described (Frokjaer-Jensen et al., 2008). Integrant lines were outcrossed at least three times.

For examination of *let-7* activity, reporter worms were subjected to RNAi by feeding as for the suppressor screen and hypodermal differentiation assay. Fluorescence intensity was compared to the empty vector control after 32 h incubation at 25 °C using a Leica MZ16 FA fluorescence dissecting microscope. Repression of the reporter was scored independently by two observers for penetrance and degree of repression. Scores for the *lin-41* 3′ UTR and the control *unc-54* 3′UTR reporters were compared to identify positive hits. Selected suppressors (Results) were imaged further on a Zeiss Z-1 microscope with Zeiss Axiovision software using equal exposure times.

To assess regulation of *cdk-1* and *cdc-25.2* 3′ UTR reporter transgenes by *let-7*, synchronized worms were grown for 36 h at 25 °C on plates. Worms were observed on a Zeiss Z-1 microscope with Axiovision software using Nomarski DIC and fluorescence microscopy.

Gene expression profiling

For microarray analysis synchronized L1 larvae were grown at 25 °C, the restrictive temperature of the temperature-sensitive sterile *glp-4(bn2)* allele (Beanan and Strome, 1992), to L4 stage (33 and 34 h for *glp-4(bn2)* and *glp-4(bn2)*; *let-7(mn112)*, respectively, to adjust for a minor growth delay of *let-7* mutant animals) and harvested in TRI Reagent (MRC). RNA was isolated according to the

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manufacturer's instructions. Total RNA (300 ng) was converted to cDNA and amplified with 1 cycle of IVT using the Affymetrix GeneChip WT Amplified Double Stranded cDNA Synthesis Kit, fragmented using the Affymetrix GeneChip WT Double-Stranded DNA Terminal Labeling Kit, and Biotin labeled using the GeneChip WT Genechip WT Terminal Labeling Kit. 7.5 μg of labeled double-stranded cDNA was hybridized to *C. elegans* tiling arrays for 16 h. Scanning was performed with Affymetrix GCC Scan Control v. 3.0.0.1214 on a GeneChip Scanner 3000 with an autoloader. All sequencing data generated for this study have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE52910 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE52910).

Raw data CEL files from tiling arrays were processed in R using a bioconductor and the packages tilingArray and preprocessCore. The arrays were RMA background corrected and log 2 transformed on the oligo level using the following command:

expr <- log 2(rma.background.correct(exprs(readCel2eSet(filenames, rotated=TRUE)))). We mapped the oligos from the tiling array (bpmap file from www.affymetrix.com) to the *C. elegans* genome assembly ce6 (www.genome.ucsc.edu) using bowtie allowing no error and unique mapping position. Expression levels for individual transcripts were calculated by intersecting the genomic positions of the oligonucleotides with transcript annotation (WormBase WS190) and averaging the intensity of the respective oligonucleotides.

miRNA target enrichment analysis

In order to test the identified suppressors of *let-7(n2853)* for enrichment of miRNA targets, ALG-1 binding site locations of L4 stage worms (Zisoulis et al., 2010) were downloaded from the *C. elegans* version ce6 (May 2008) UCSC genome annotation database (http://hgdownload.soe.ucsc.edu/goldenPath/ce6/database/).

Gene annotations were previously downloaded from WormBase for the *C. elegans* genome version WS190, corresponding to UCSC version ce6. ALG-1 binding sites were assigned to the nearest annotated transcript using the BedTools intersect utility (Quinlan and Hall, 2010), and 3217 unique gene IDs were extracted from the resulting list. The number of genes expressed during L4 stage was calculated based on published expression data (Hendriks et al., 2014). To this end, samples from a total of 9 time points of continuous development (28–36 h) were first normalized for library size, averaged and log 2 transformed. We used a cutoff of 4 (in log 2 space) to separate expressed from non-expressed genes based on the bimodal expression distribution, yielding 15,179 expressed genes. An enrichment of putative miRNA targets among the different classes of miRNA suppressors (see main text) was tested by comparison against this baseline frequency of 0.212 (3217 of 15,179 genes) miRNA targets per expressed gene using a hypergeometric test.

Results and discussion

A genome-wide RNAi screen identifies 201 suppressors of the let-7 (n2853) lethality phenotype

To study the *let-7* regulatory network on a global level, we sought suppressors of the temperature-sensitive (*ts*) *let-7(n2853)* vulval bursting phenotype in a genome-wide, RNAi-based screen.

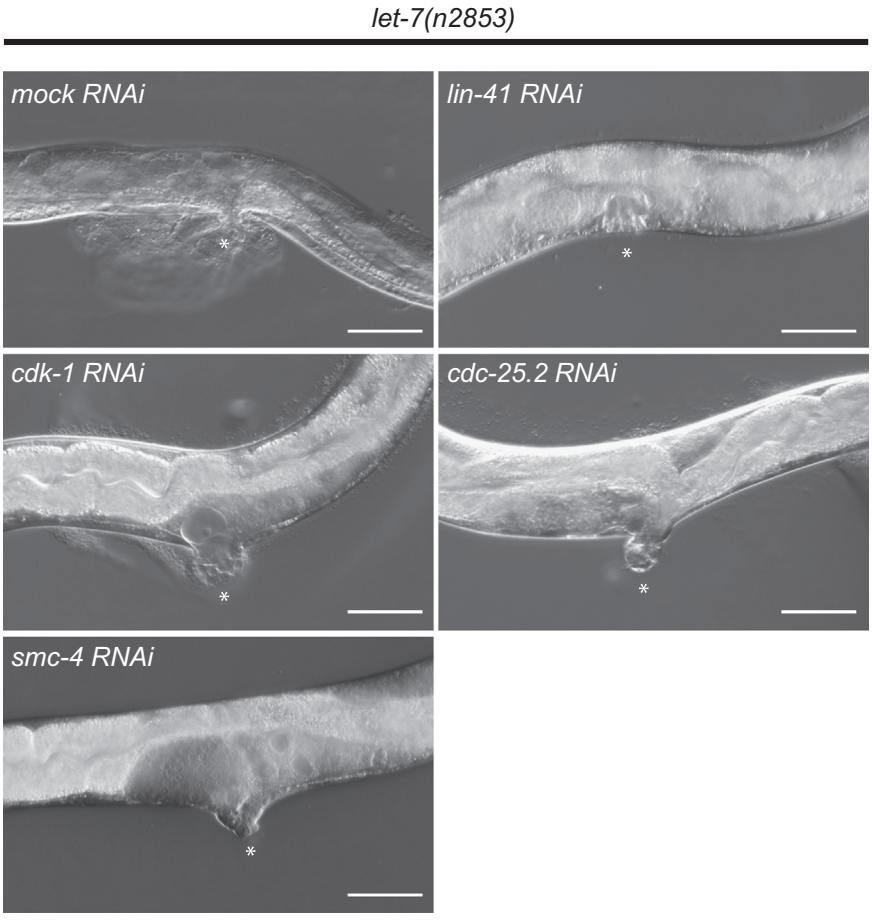

Fig. 1. A genome-wide RNAi screen for suppressors of *let-7(n2853)* bursting. Knock-down of the indicated suppressors by RNAi rescues bursting of *let-7(n2853)* worms. Vulvae are marked with asterisks. Scale bar indicates 50 μm.

let-7(n2853) worms carry a G-to-A point mutation in the seed sequence of the mature miRNA, leading to impaired binding and repression of *let-7* targets as well as reduced expression of the mature miRNA (Reinhart et al., 2000). The resulting vulval bursting phenotype at the L/A transition is highly penetrant at the restrictive temperature of 25 °C. At 15 °C, *let-7(n2853)ts* animals are viable, but seam cells continue to divide and fail to differentiate (Reinhart et al., 2000), whereas at an intermediate temperature, 20 °C, lethality occurs but at reduced penetrance (Großhans et al., 2005).

In a pilot experiment, we had previously used RNAi by feeding against genes on *C. elegans* chromosome I to identify suppressor genes of the *let-7(n2853)* lethality at 20 °C and 25 °C (Ding et al. 2008). To expand the screen from these 2400 genes to a genome-wide scale, we complemented the "Ahringer library" with select RNAi clones from the "Vidal library" to cover >90% of *C. elegans* genes (Kamath et al., 2003; Rual et al., 2004). Moreover, we streamlined the screening procedure further by performing it in liquid medium, and at only one temperature, 25 °C, followed by rescreening of primary candidates on RNAi plates at both 20 °C and 25 °C. Plasmids from bacteria scoring positive in the second round of screening were isolated, sequenced, and retransformed into bacteria, which were then utilized for a final round of testing for suppression. Through these three rounds of testing, we validated 201 genes as suppressors of *let-7* lethality that were capable of restoring viability of at least 20% of the worms in one or both conditions (Fig. 1 and Table S1). Note that some suppressed animals retained a protruding vulva phenotype, reflecting incomplete suppression or a separate vulval defect due to depletion of the targeted gene (see also below). Our screen also covered the previously screened chromosome I (Ding et al., 2008), permitting us to compare the two datasets. We found that we had rediscovered a high 78% of the candidates identified in the previous study (Table S1), which demonstrates the interactions to be robust and reproducible even under distinct screening conditions.

Modulation of let-7 function by suppressors of vulval bursting

The list of 201 suppressors also contained five out of 61 genes previously identified as enhancers of vulval bursting associated with the weak *let-7(mg279)* hypomorphic allele in a total of 17,900 genes tested by RNAi (Parry et al., 2007). Although few, this constitutes a 7.3-fold enrichment over background (p-Value$=6\times10^{-4}$, hypergeometrical test). Possibly, the activity levels of these specific genes need to be very tightly regulated. Hence, their presumably greater depletion in the RNAi-sensitized strain used in the previous study (Parry et al., 2007) might have resulted in different effects from those seen here. Regardless of this possibility, the finding indicated a need for a better understanding of the suppressor genes. As a first step, we sought to determine whether any of the *let-7(n2853)* suppressor genes were negative regulators of *let-7*-mediated gene silencing. Hence, we developed a GFP-based *let-7* target reporter system to directly analyze *let-7*-activity in hypodermal cells in vivo. We fused the hypodermis-specific *wrt-2* promoter (Aspöck et al., 1999) to a gene encoding a destabilized nuclear GFP (GFP-H2B-PEST) followed by the 3′UTR of *lin-41*, which we chose as the best-characterized target of *let-7* (Vella et al., 2004). In addition to this reporter, which we termed *pREP_lin-41*, we generated control reporters, *pREP_unc54* and *pREP_lin41ΔLCS*, which contained the unregulated *unc-54* 3′UTR and a *lin-41* 3′UTR lacking a 98nt fragment required for *let-7*-mediated regulation (Vella et al., 2004), respectively. All three transgenes were integrated into the same genomic site in single copy through Mos1 transposon-mediated single copy transgene integration (MosSCI) (Frokjaer-Jensen et al., 2008).

The reporter system faithfully recapitulated *let-7*-mediated regulation: all three reporters were highly expressed in the hypodermis of early wild-type larvae. Subsequently, *pREP_lin41*, but not *pREP_unc54* or *pREP_lin41ΔLCS,* showed repression starting during L4 larval stage (Fig. 2A and data not shown). This correlates well with the accumulation of *let-7* during the L4 stage (Reinhart et al., 2000). The differences in expression between the control reporters and *pREP_lin-41* increased further when adult animals were examined. In old adults, even the signal from the control reporters declined substantially, presumably reflecting decreased promoter activity. We confirmed that repression of *pREP_lin41* depended on *let-7* by crossing the reporters into *let-7(n2853)* mutant animals. This resulted in elevated *pREP_lin41* expression levels in L4 and adult stage animals relative to their wild-type counterparts, whereas expression of *pREP_unc54* and *pREP_lin41ΔLCS* remained unaffected (Fig. 2A and data not shown).

Transcriptional profiling data from our lab recently revealed periodic *wrt-2* mRNA accumulation during larval development (Hendriks et al., 2014), and the *pREP_unc54* reporter indeed exhibited increased *wrt-2* promoter activity towards the end of the L4 stage. As the fluctuation of GFP was less than that of the endogenous *wrt-2* mRNA, we could control for this potential source of variability in *pREP_lin41* experiments by the examination of worms carrying the *pREP_unc54* control transgene. Furthermore, a reporter carrying the 3′UTR of the *let-7* target *daf-12* (Großhans et al., 2005) (*pREP_daf12*) was used to test independently for restoration of *let-7* activity. Analyzing the full set of our identified suppressors, we found 73 genes to restore repression of a *let-7* target reporter in the *let-7(n2853)* background while showing no or modest repression of the control 3′UTR upon RNAi ('target reporter positives', Fig. 2B and Table S2).

A subset of the suppressors affect let-7-dependent hypodermis differentiation

It was conceivable that some suppressors modulated vulval development and/or morphogenesis in a *let-7*-independent manner, thus preventing bursting indirectly. Consistent with this notion, we frequently observed protruding vulva (Pvl) phenotypes upon suppressor RNAi on wild-type as well as on *let-7(n2853)* animals (Table S1). Therefore, we wished to examine suppression of another *let-7* mutant phenotype, outside the vulva. We utilized a previously established *Pcol-19::gfp* reporter (Abrahante et al., 1998) to examine whether hypodermal cell differentiation was also restored upon depletion of the suppressor genes. Transcription of *col-19*, an adult-specific cuticular collagen gene, requires the zinc-finger transcription factor LIN-29 (Rougvie and Ambros, 1995; Liu et al., 1995) (Fig. 3A), which, however, does not accumulate in *let-7(n2853)* mutant animals (Reinhart et al., 2000). Accordingly, *Pcol-19::gfp* is not expressed in *let-7* mutant animals (Fig. 3B). By contrast, depletion of 102 of the 201 *let-7* suppressor genes resulted in GFP accumulation in adult animals ('col-19 positives', Fig. 3B and Table S3). Hence, depletion of these genes restores at least some aspect of hypodermal cell differentiation, further supporting their function in the heterochronic pathway.

let-7 suppressor genes can be grouped into four functional classes

Taken together, the results of the three different assays that measure restoration of viability, *let-7* target gene repression, and restoration of seam cell differentiation, yield four different groups of suppressor genes (Fig. S2). 'Suppressor-only' genes are positive for restoration of viability, but none of the other assays.

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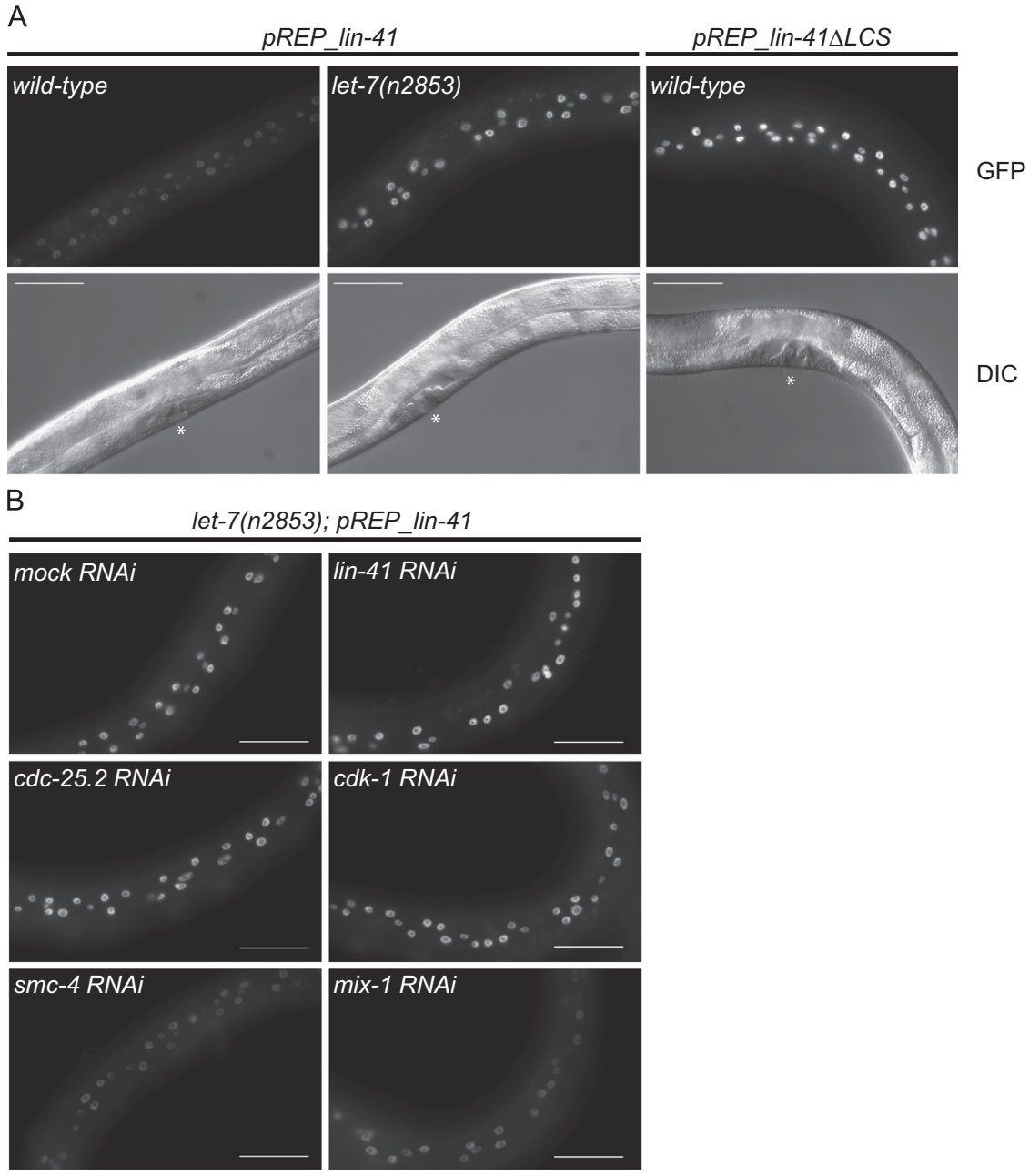

Fig. 2. *let-7* suppressor RNAi restores repression of a *let-7* target reporter. (A) Repression of a *let-7* target reporter (*Pwrt-2::gfp::lin-41 3′UTR*, "*pREP_lin-41*") in late L4 worms depends on *let-7* and is lost upon mutation of the *let-7* complementary sites (*pREP_lin-41ΔLCS*). Vulvae are marked with asterisks. (B) GFP intensity in *pREP_lin-41, let-7 (n2853)* worms subjected to the indicated RNAi; pictures were taken at the young adult stage. RNAi against *smc-4* and *mix-1*, but not against the other genes, causes repression of the reporter. Scale bar indicates 50 μm.

These genes may be enriched for false positive hits, modulate *let-7* functions that are currently unknown, or act in tissues other than the hypodermis.

The three other classes contain genes that are all positive for restoration of viability, and additionally one or both of the other assays. Thus, 'target reporter-only' genes are positive for target reporter repression, but not for *Pcol-19::gfp* expression. In a linear model, where increased *let-7* target repression would proportionally enhance *let-7*-dependent cellular differentiation, these genes may be false positive hits. However, it seems equally possible that modulation of the developmental phenotype, measured by *Pcol-19::gfp* expression, needs restoration of target gene repression beyond a certain threshold, and/or that the sensitivities of the two assays differ. Finally, the genes in this class may only alter activity of some *let-7* target genes, with hypodermis differentiation depending at least in part on some targets whose activity we have not measured here.

Genes in the 'col-19-only' group affect *Pcol-19::gfp* expression without apparent effects on *let-7* target gene silencing. These genes might act downstream of, or in parallel to, *let-7*, potentially as direct *let-7* targets or indirect effectors, and we provide a detailed dissection of one example below.

Finally, a group of 36 genes scored positive in both the target reporter and the *col-19* expression assays (Table 1) and constitute the 'double-positive' class. Although the mechanisms by which these genes function remain to be established, they are strong candidates for modulators of *let-7* activity. Notably, this list includes all five members of the *C. elegans* condensin II complex, namely *smc-4*, *mix-1*, *kle-2*, *capg-2*, and *hcp-6* (Csankovszki et al., 2009) as well as *plk-1*, the *C. elegans* orthologue of Polo-like kinase

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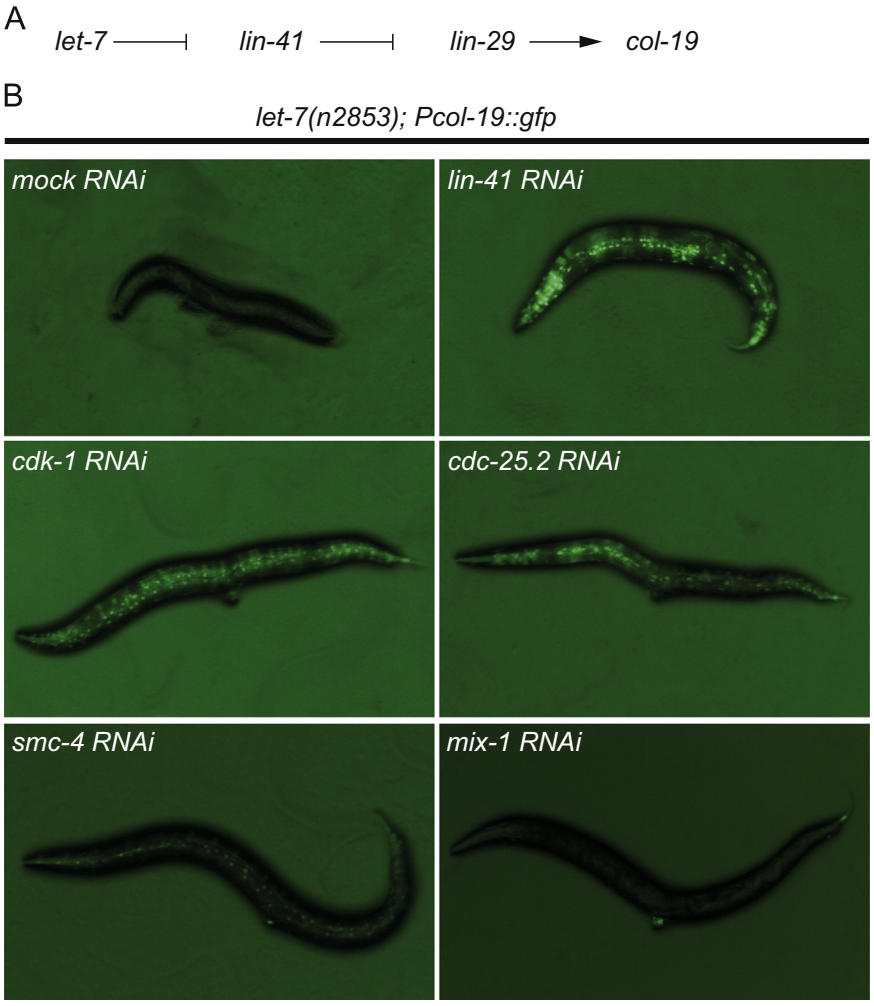

Fig. 3. RNAi of *let-7(n2853)* suppressors restores hypodermis differentiation. (A) Activation of the adult-specific *col-19* promoter is controlled by *let-7* through activation of the transcription factor LIN-29. (B) Expression of *col-19::gfp* in *let-7(n2853)* worms subjected to the indicated RNAi; pictures were taken at the young adult stage (100 × magnification). RNAi against *cdk-1* and *cdc-25.2* but not against *smc-4* or *mix-1* causes upregulation of the reporter.

(Ouyang et al., 1999), a known regulator of condensins in human HeLa cells (Abe et al., 2011). RNAi of the condensin II complex has been shown to result in chromosome condensation and segregation defects both in mitosis and meiosis (Hagstrom et al., 2002; Stear and Roth, 2002), but in addition to its structural functions, the complex was reported to bind to interphase chromatin in *C. elegans* where it acts as a transcriptional repressor (Kranz et al., 2013). Although we have currently no mechanistic explanation for the ability of condensin II to modulate *let-7* activity, the identification of this entire complex further corroborates the robustness of our analysis, and makes condensin II a particularly interesting candidate miRNA pathway factor.

Most novel suppressors are unlikely to be direct let-7 targets

Zisoulis et al. (2010) previously identified candidate miRNA targets through their association with the miRNA Argonaute protein ALG-1. Interestingly, we found that 81 out of 201 suppressors as well as 41 out of the 102 'col-19 positive' suppressors were also bound by ALG-1. This represents a moderate enrichment of 1.9-fold for both classes compared to the 3217 ALG-1 bound mRNAs in a total of 15,179 genes expressed in L4 (total suppressors: p-Value $= 5.1 \times 10^{-10}$, 'col-19 positives': p-Value $= 9.9 \times 10^{-6}$, hypergeometric test; see *Methods*). To determine whether a subset of these genes was indeed regulated by *let-7*, we compared gene expression patterns of wild-type and *let-7(mn112)* null mutant worms at the late L4 stage using *C. elegans* tiling arrays. Because *let-7* activity has not been reported in the germline, we performed these experiments in germline-less *glp-4(bn2)* mutant animals (Beanan and Strome, 1992), to examine gene expression levels specifically in somatic tissues (Fig. 4 and S1). Analysis of the data did reveal robust overexpression of the published *let-7* targets *lin-41* (4.17 fold) and *daf-12* (2.1 fold) in *let-7(mn112)* compared to wild-type worms. By contrast, most of the novel suppressors did not change in *let-7* mutant worms. This finding implies that, consistent with the moderate enrichment of ALG-1 binders, the majority of *let-7* suppressors are not direct *let-7* targets. This notion is also supported by our recent finding that vulval bursting of *let-7* mutant animals is explained by dysregulation of only LIN-41 (Ecsedi et al., 2015). Alternatively, some of these genes may either be *let-7* targets regulated through mechanisms that do not involve substantial mRNA degradation, e.g., translational control, or their downregulation may occur in only a subset of tissues, making detection impossible in whole worm RNA.

let-7 regulates CDK-1 expression in a LIN-29-dependent manner

Since gene expression profiling failed to reveal new *let-7* targets or downstream effectors, we sought to find specific examples of such genes by examining the 'col-19-only' suppressors. Previous work on cultured cells revealed that *let-7* targets include a cyclin-dependent kinase, CDK6, and a CDK-regulating phosphatase, CDC25A (Johnson

Table 1
List of suppressors positive for both target reporter repression and hypodermis differentiation assay ('double-positive' genes). Shown are all genes which upon RNAi rescue both adult hypodermis formation (*Pcol-19::gfp* reporter assay) as well as repression of a *let-7* target reporter (*Pwrt-2::gfp-H2B-PEST::lin-41-3′UTR* or *Pwrt-2::gfp-H2B-PEST::daf-12-3′UTR*) in *let-7(n2853)* animals. *Pcol-19::gfp* reporter assay: weak (+), medium (++) or strong (+++) activation of GFP upon RNAi. *let-7* target reporter: weak (+), medium (++) or strong (+++) repression of GFP upon RNAi.

	Predicted gene	*col-19* activation				Target reporter repression			Function
		25 °C		20 °C					
		49 h	58 h	56 h	72 h	*lin-41*	*daf-12*	*unc-54* (ctrl.)	
Cell cycle/chromosome maintenance and segregation	hcp-6	–	++	–	+++	++	+++	+	Condensin II subunit
	capg-2	–	++	–	+++	++	+	–	Condensin II subunit
	kle-2	+	+++	–	+++	+	–	–	Condensin II subunit
	smc-4	–	++	–	++	++	–	–	Condensin II subunit
	mix-1	–	–	–	++	+++	–	–	Condensin II subunit
	scc-3	+	+++	–	+++	–	+	–	Cohesin subunit
	cyb-3	+	++	–	+++	++	–	–	Cyclin B
	plk-1	+	++	–	+++	+	+	–	Polo-like kinase
	knl-2	–	++	–	++	+++	++	–	Kinetochore associated
	him-1	++	+++	–	+++	+	–	–	Structural maintenance of chromosome family
DNA/replication	lig-1	+	++	–	+++	+	–	–	DNA ligase
	Y47D3A.29	–	+	–	–	–	++	–	DNA polymerase alpha subunit
	pri-1	–	++	–	++	+++	++	–	DNA primase
	ruvb-2	–	+	–	+	++	+	–	Recombination protein homolog
	rpa-1	–	+	–	+	–	++	–	Replication protein A homolog
mRNA biogenesis	rpb-7	–	+	–	+	++	+	–	RNA Pol II subunit
	cpsf-2	–	–	–	+	++	++	–	Cleavage and polyadenylation specificity factor
	symk-1	–	+	–	–	+	+	–	Cleavage and polyadenylation factor
	prp-21	+	+	–	++	–	+	–	Splicing factor related
	uaf-1	–	–	–	++	++	++	–	Splicing factor related
Ribosome biogenesis	C37H5.5	–	+	–	+	–	+	–	Nucleolar complex protein 3 homolog
	C47E12.7	–	+	–	–	–	+	–	Ribosomal RNA processing protein 1 homolog
	K12H4.3	–	+	–	–	–	+	–	Ribosome biogenesis protein BRX1 homolog
Nuclear transport	npp-3	–	+	–	+	–	++	–	Nuclear pore protein
	npp-9	–	+	–	–	++	++	–	Nuclear pore protein
	npp-6	+	++	–	+	+++	+++	+	Nuclear pore protein
	xpo-2	+	+	–	++	+	+	–	Nuclear export receptor
Other	aco-2	+	++	–	++	++	–	–	Aconitase
	pyp-1	+	+	+	++	+	–	–	Pyrophosphatase, nucleosome remodeling?
	ani-1	+	+	–	++	+	–	–	Actin binding protein
	dut-1	–	–	–	++	+	–	–	DeoxyUTPase
	toe-1	–	–	–	+	–	+	–	Target of ERK kinase MPK-1
	nhr-25	+++	+++	+++	+++	+	+	–	Nuclear hormone receptor
	T06E6.1	–	–	–	+	–	+	–	
	F44G4.1	–	–	–	+	+	–	–	
	C16A3.4	–	+	–	–	–	++	–	
Control	hda-1	–	–	–	–	–	–	–	Randomly chosen 'suppressor-only'

et al., 2007). Although the functional relevance of these interactions remained unclear, *let-7* has a conserved function in regulation of cell proliferation (Büssing et al., 2008). We were thus intrigued by the identification of *ncc-1*/*cdk-1* (Mori et al., 1994; Boxem et al., 1999) and its activating phosphatase *cdc-25.2* (Kim et al., 2010) among this class of suppressors of vulval bursting. To place the two genes in the pathway, we tested whether their depletion suppressed also vulval bursting caused by the *let-7(mn112)* null mutation, which we found to be the case. We observed 97% rescue of bursting for *cdk-1* RNAi and 99% rescue for *cdc-25.2* ($n > 200$ each). About half of the surviving worms were vulvaless (data not shown). Although suppression of bursting might therefore, in part, be indirect, restoration of *col-19::gfp* expression in the hypodermis supported specificity of the genetic interaction (Fig. 3, Table S3). To examine this further, we analyzed the formation of adult alae in *let-7(mn112)* mutant animals. Strikingly, whereas only 9% ($n=32$) of *let-7(mn112)* animals on mock RNAi displayed any alae, 51% ($n=47$) of animals on *cdk-1(RNAi)* and 41% ($n=27$) of animals on *cdc-25.2(RNAi)* did. Similar to the *lin-41(RNAi)* positive control, knockdown of *cdk-1* and *cdc-25.2* virtually always resulted in partial, rather than complete alae, whereas the occasional animals on mock RNAi typically exhibited weak but complete alae. Hence, *cdk-1* and *cdc-25.2* exhibit hallmarks of a downstream effector of *let-7*.

Based on these results it seemed possible that *cdk-1* and *cdc-25.2* were direct targets of *let-7*. Because *let-7* targets that are regulated in a tissue-specific manner and/or through translational repression might not be evident from whole animal gene expression studies by microarray, we generated *cdk-1* and *cdc-25.2* 3′ UTR reporters to assess their potential for regulation by *let-7*. When we analyzed these reporters, *pREP_cdk1* and *pREP_cdc-25.2*, respectively, we found them both to be repressed in L4 stage animals in both seam cells and the hyp7 syncytium relative to the unregulated *pREP_unc54* control reporter (Fig. 5). For *pREP_cdk1* this repression was more pronounced in hyp7 than the seam, whereas the opposite was true for *pREP_cdc-25.2*. However, whereas the positive control *pREP_lin-41* was efficiently derepressed in the *let-7(n2853)* mutant background, this was not observed for *pREP_cdk-1* and *pREP_cdc25.2* in either tissue. We conclude that although the 3′ UTRs of these two mitotic genes

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might confer post-transcriptional repression at the L4 stage, when *let-7* is present, this seems unlikely to be a consequence of *let-7* function.

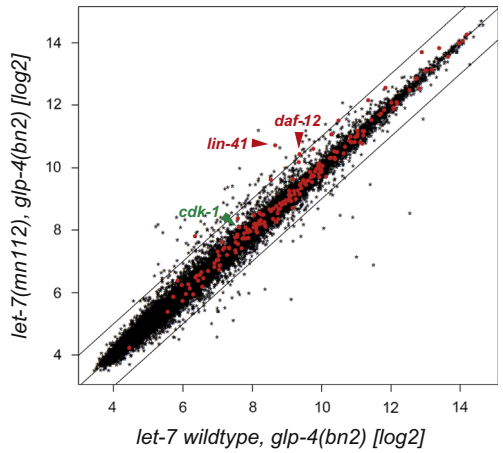

Fig. 4. Expression levels of novel *let-7* suppressors are not affected in *let-7* mutants. Microarray analysis of somatic gene expression in *let-7(mn112)* null mutant in germline-less *glp-4(bn2)* animals shows no changes in mRNA levels for genes identified as suppressors of the *let-7(n2853)* bursting phenotype (marked in red). The known *let-7* targets *lin-41* and *daf-12* are indicated in red for reference, *cdk-1* in green.

We therefore wondered if *cdk-1* functioned further downstream of *let-7* in the heterochronic pathway. We utilized a previously published *cdk-1::gfp* single copy-integrated transgene, which drives expression of a functional fusion protein from the native *cdk-1* promoter (Shirayama et al., 2012), to examine the effect of *let-7* on CDK-1 accumulation. We observed that CDK-1/GFP was present in early L4-stage seam cells, but that its levels declined rapidly upon entry into adulthood (Fig. 6A). However, down-regulation was impaired in *let-7(n2853)* mutant animals where CDK-1/GFP was well visible in the seam cell cytoplasm and, prominently, nucleus. To understand better why CDK-1/GFP protein levels responded so strongly to loss of *let-7* activity although *let-7* did not appear to repress it directly, we tested whether *cdk-1::gfp* expression was modulated by the downstream effector LIN-29. Indeed, knock-down of *lin-29* by RNAi resulted in elevated levels and redistribution of CDK-1/GFP, similar to the effect of *let-7(n2853)* (Fig. 6B). Finally, this was also observed for RNAi of *mab-10* (Fig. 6B), a transcription co-factor that acts in concert with LIN-29 to promote differentiation of the hypodermis (Harris and Horvitz, 2011). Thus, we conclude that *let-7* regulates *cdk-1* indirectly, in a manner that requires the LIN-29 transcription factor.

Conclusion

Using a genome-wide screen, we have identified and characterized here >200 suppressors of *let-7* mutant phenotypes. In combination

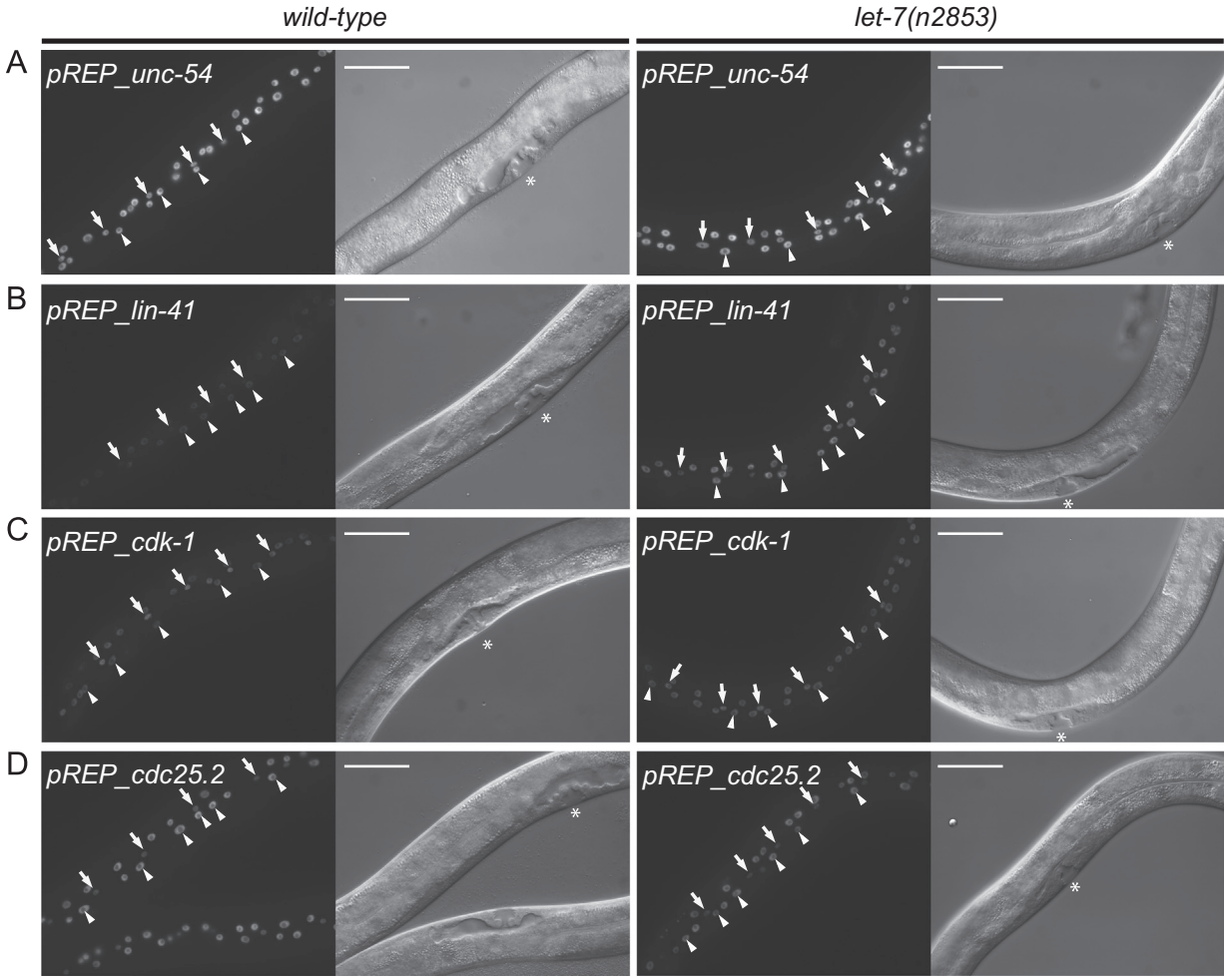

Fig. 5. The 3′UTRs of *cdk-1* and *cdc-25.2* do not confer *let-7*-dependent regulation. (A) A hypodermis specific target reporter (*wrt-2* promoter) containing *gfp* fused to the unregulated *unc-54* 3′UTR (*pREP_unc-54*) is expressed both in wild-type and *let-7(n2853)* background at the late L4 stage. (B–D) The reporter containing the *lin-41* 3′UTR (*pREP_lin-41*) is repressed in a *let-7* dependent manner (B) while repression of reporters carrying the *cdk-1* (*pREP_cdk-1*, C) or *cdc-25.2* 3′UTR (*pREP_cdc-25.2*, D) in wild-type worms is less extensive and persists in the *let-7(n2853)* background. Vulvae are marked with asterisks. Scale bar indicates 50 μm.

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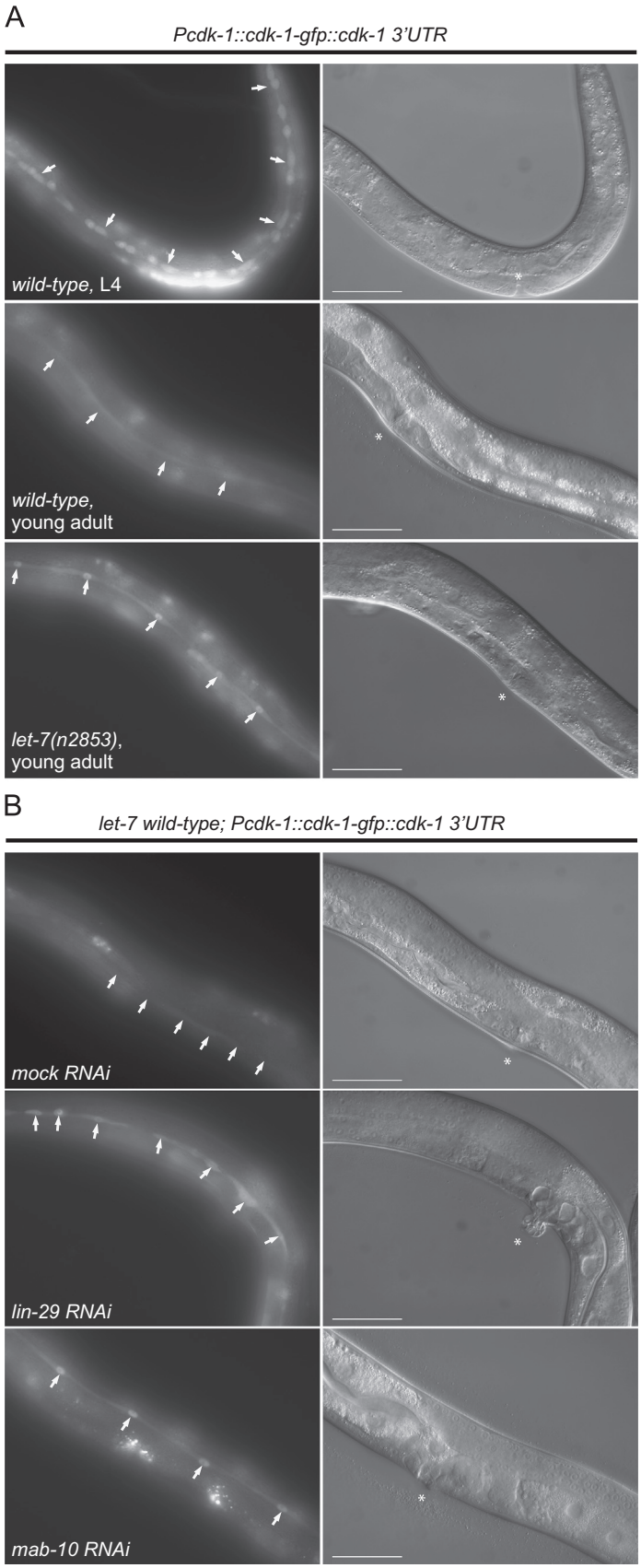

Fig. 6. Repression of *cdk-1::gfp* depends on LIN-29 and MAB-10 (A) Expression of *cdk-1::gfp* from the *cdk-1* promoter can be observed in seam cells (arrows) until the L4 stage. GFP levels decrease during L4 stage in wild-type background. *let-7(n2853)* mutant animals continue to express *cdk-1::gfp* in adult stage. (B) Downregulation of *cdk-1::gfp* in wild-type worms is lost upon RNAi-mediated knockdown of *lin-29* or *mab-10*. Vulvae are marked with asterisks. Scale bar indicates 50 μm.

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with previous work using genetic enhancer screening (Parry et al., 2007) and genomics analysis (Hunter et al., 2013) of *let-7* mutant strains, a comprehensive picture of the genetic interactome of *let-7* becomes available, promoting a better understanding of this model miRNA and key developmental regulator. Thus, among the newly identified suppressors, we consider the 'col-19 positive' and the 'double-positive' genes to be of particular interest for studies of the heterochronic pathway and miRNA function and regulation, respectively. Our analysis of CDK-1, which we identified as a putative effector of *let-7* based on its placement in the 'col-19-only' class, illustrates the utility of this approach: whereas CDK-1 was unremarkable in transcriptome analysis, its proficiency in suppressing both *let-7* mutant lethality and hypodermis differentiation defects suggested a functionally relevant interaction with *let-7*, prompting us to test and confirm its regulation by *let-7* and via LIN-29 through more specific means.

As *let-7* controls cell proliferation, it must, at some level, interface with the cell cycle machinery. However, an interaction with the mitotic CDK-1 is unexpected, as the exit of seam cells from proliferation is expected to occur in G1, not G2/M. Therefore, based on the facts that LIN-29 also regulates the cell cycle inhibitor CKI-1 (Hong et al., 1998) and that additional cell cycle genes occur among the 'col-19-only' and the 'double-positive' suppressor genes, we speculate that repression of CDK-1 might be part of a larger program of repression of cell cycle genes during exit of seam cells from proliferation. The observation that CDK functions are plastic such that CDK1 can partially substitute for other CDKs during mouse embryonic development (Santamaria et al., 2007) might explain the need for its repression.

Interestingly, depletion of CDK-1 not only prevents seam cell overproliferation in *let-7* mutant animals, but also promotes hypodermis differentiation by two criteria, expression of *Pcol-19::gfp*, and formation of adult alae. Conceivably, this reflects a tight coupling of cell proliferation and differentiation in the seam so that differentiation ensues when proliferation is blocked. However, we note that *cdk-1(RNAi)* also promotes *Pcol-19::gfp* expression in the postmitotic hyp7, potentially reflecting a more direct role on differentiation. Moreover, we find that even proliferating seam cells can express *Pcol-19::gfp*. For instance, we observed that depletion of *rnr-1*, which codes for the large subunit ribonucleotide reductase, promotes expression of *Pcol-19::gfp* without preventing seam cell overproliferation. Thus, when scored using the seam cell-specific *scm::gfp* marker to visualize seam cells (Koh and Rothman, 2001), *let-7(n2853)* mutant animals exposed to mock or *rnr-1(RNAi)* have a comparable number of seam cells at the young adult stage, i.e., an average of 23.6 cells ($n=22$) and 22.5 ($n=21$), respectively, per side, well above the wild-type 16. Yet *rnr-1(RNAi)* promotes expression of *col-19::gfp* (Table S3). This suggests that a potential coupling between cell cycle exit and differentiation, if it exists, would be unidirectional.

Finally, the observation that the 'double-positive' group of supressors contains a number of genes encoding structural components of chromosomes and cell cycle factors, provides a further illustration of the apparently complex relationship between *let-7* function in the heterochronic pathway and the cell cycle. We propose that our comprehensive genetic screen has thus opened a new door to a deeper understanding of *let-7* and miRNA function more generally.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2015.02.013.

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